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(57) Abstract

The present invention relates to peptides and proteins such as receptors that bind angiogenesis–related proteins Angiostatin protein and/or Endostatin protein. Peptides and proteins of the present invention can be isolated from body fluids including blood or urine, or can be synthesized by recombinant, enzymatic or chemical methods. The peptides are particularly important for identifying receptors of angiogenesis–related proteins, as well as for identifying other proteins that regulate, transport and otherwise interact with angiogenesis–related proteins. The present invention in particular relates to laminin protein as a putative receptor for Angiostatin protein and tropomyosin as a putative Endostatin protein.

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COMPOSITIONS AND METHODS OF USING PROTEINS AND PEPTIDES THAT BIND ANGIOGENESIS-INHIBITING PROTEINS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to United States Patent Application Serial No. 09/206,059 filed December 4, 1998. This application is also related to United States Provisional Patent Application Serial No. 60/150,938 filed August 26, 1999.

FIELD OF THE INVENTION

The present invention relates to peptides and proteins that bind angiogenesis-related proteins, such as Angiostatin protein or Endostatin protein. Peptides and proteins of the present invention can be isolated from body fluids and tissues including blood or urine, or can be synthesized by recombinant, enzymatic or chemical methods. In addition, the present invention relates to the use of such binding molecules in diagnostic assays and kits for protein measurement, histochemical kits for protein localization, nucleotide sequences coding for protein, and molecular probes for monitoring protein biosynthesis, wherein the protein is related to angiogenesis.

BACKGROUND OF THE INVENTION

As used herein, the term "angiogenesis" means the generation of new blood vessels into a tissue or organ. Under normal physiological conditions, humans or animals undergo angiogenesis only in very specific restricted situations. For example, angiogenesis is normally observed in wound healing, fetal and embryonal development and formation of the corpus luteum, endometrium and placenta. The term "endothelium"

means a thin layer of flat epithelial cells that lines serous cavities, lymph vessels. and blood vessels.

Both controlled and uncontrolled angiogenesis are thought to proceed in a similar manner. Endothelial cells and pericytes, surrounded by a basement membrane, form capillary blood vessels. Angiogenesis begins with the erosion of the basement membrane by enzymes released by endothelial cells and leukocytes. The endothelial cells, which line the lumen of blood vessels, then protrude through the basement membrane. Angiogenic stimulants induce the endothelial cells to migrate through the eroded basement membrane. The migrating cells form a "sprout" off the parent blood vessel, where the endothelial cells undergo mitosis and proliferate. The endothelial sprouts merge with each other to form capillary loops thereby creating the new blood vessel.

Persistent, unregulated angiogenesis occurs in a multiplicity of disease states, tumor metastasis and abnormal growth by endothelial cells; furthermore. unregulated angiogenesis also supports the pathological damage seen in these conditions. The diverse pathological disease states in which unregulated angiogenesis is present have been grouped together as angiogenic-dependent or angiogenic-associated diseases.

The hypothesis that tumor growth is angiogenesis-dependent was first proposed in 1971. (Folkman J., Tumor angiogenesis: Therapeutic implications., *N. Engl. Jour. Med.* 285:1182 1186. 1971) In its simplest terms it states: "Once tumor 'take' has occurred, every increase in tumor cell population must be preceded by an increase in new capillaries converging on the tumor." Tumor "take" is currently understood to indicate a prevascular phase of tumor growth in which a population of tumor cells occupying a few cubic millimeters volume and not exceeding a few million cells, can survive on existing host microvessels. Expansion of tumor volume beyond this phase requires the induction of new capillary blood vessels. For example, pulmonary micrometastases in the early prevascular phase in mice would be undetectable except by high power microscopy on histological sections.

It is clear that angiogenesis plays a major role in the metastasis of a cancer. If this angiogenic activity could be repressed or eliminated, then the tumor, although present, would not grow. In the disease state, prevention of angiogenesis could avert the damage caused by the invasion of the new microvascular system. Therapies directed at control of the angiogenic processes could lead to the abrogation or mitigation of these diseases.

One example of a disease mediated by angiogenesis is ocular neovascular disease. This disease is characterized by invasion of new blood vessels into the structures of the eye such as the retina or cornea. It is the most common cause of blindness and is involved in approximately twenty eye diseases. In age-related macular degeneration, the associated visual problems are caused by an ingrowth of chorioidal capillaries through defects in Bruch's membrane with proliferation of fibrovascular tissue beneath the retinal pigment epithelium. Angiogenic damage is also associated with diabetic retinopathy, retinopathy of prematurity, corneal graft rejection, neovascular glaucoma and retrolental fibroplasia. Other diseases associated with corneal neovascularization include, but are not limited to, epidemic keratoconjunctivitis, Vitamin A deficiency, contact lens overwear, osteoporosis, atopic keratitis, superior limbic keratitis, pterygium keratitis sicca, sjogrens, acne rosacea, phylectenulosis, syphilis, Mycobacteria infections, lipid degeneration, chemical burns, bacterial ulcers, fungal ulcers, Herpes simplex infections, Herpes zoster infections, protozoan infections, Kaposi sarcoma, Mooren ulcer, Terrien's marginal degeneration, mariginal keratolysis, rheumatoid arthritis, systemic lupus, polyarteritis, trauma, Wegener's sarcoidosis, Scleritis, Steven's Johnson disease, periphigoid radial keratotomy, and corneal graph rejection.

Diseases associated with retinal/choroidal neovascularization include, but are not limited to, diabetic retinopathy, macular degeneration, sickle cell anemia, sarcoid, syphilis, pseudoxanthoma elasticum, Paget's disease, vein occlusion, artery occlusion, carotid obstructive disease, chronic uveitis/vitritis, mycobacterial infections, Lyme's disease, systemic lupus erythematosis, retinopathy of prematurity, Eales disease, Bechet's disease, infections causing a retinitis or choroiditis,

presumed ocular histoplasmosis, Best's disease, myopia, optic pits, Stargart's disease, pars planitis, chronic retinal detachment, hyperviscosity syndromes, toxoplasmosis, trauma and post-laser complications. Other diseases include, but are not limited to, diseases associated with rubeosis (neovascularization of the angle) and diseases caused by the abnormal proliferation of fibrovascular or fibrous tissue including all forms of proliferative vitreoretinopathy.

Another disease in which angiogenesis is believed to be involved is rheumatoid arthritis. The blood vessels in the synovial lining of the joints undergo angiogenesis. In addition to forming new vascular networks, the endothelial cells release factors and reactive oxygen species that lead to pannus growth and cartilage destruction. The factors involved in angiogenesis may actively contribute to, and help maintain, the chronically inflamed state of rheumatoid arthritis.

Factors associated with angiogenesis may also have a role in osteoarthritis. The activation of the chondrocytes by angiogenic-related factors contributes to the destruction of the joint. At a later stage, the angiogenic factors would promote new bone formation. Therapeutic intervention that prevents the bone destruction could halt the progress of the disease and provide relief for persons suffering with arthritis.

Chronic inflammation may also involve pathological angiogenesis. Such disease states as ulcerative colitis and Crohn's disease show histological changes with the ingrowth of new blood vessels into the inflamed tissues. Bartonellosis, a bacterial infection found in South America, can result in a chronic stage that is characterized by proliferation of vascular endothelial cells. Another pathological role associated with angiogenesis is found in atherosclerosis. The plaques formed within the lumen of blood vessels have been shown to have angiogenic stimulatory activity.

One of the most frequent angiogenic diseases of childhood is the hemangioma. In most cases, the tumors are benign and regress without intervention. In more severe cases, the tumors progress to large cavernous and infiltrative forms and create clinical complications. Systemic forms of hemangiomas, the hemangiomatoses, have a high mortality rate. Therapy resistant

hemangiomas exist that cannot be treated with therapeutics currently in use.

Angiogenesis is also responsible for damage found in hereditary diseases such as Osler-Weber-Rendu disease, or hereditary hemorrhagic telangiectasia. This is an inherited disease characterized by multiple small angiomas, tumors of blood or lymph vessels. The angiomas are found in the skin and mucous membranes, often accompanied by epistaxis (nosebleeds) or gastrointestinal bleeding and sometimes with pulmonary or hepatic arteriovenous fistula.

Numerous efforts have been made by researchers in the pharmaceutical industry to improve the target specificity of drugs. As is familiar to those skilled in the art, the manifestation of a disease many times involves the display of a particular cell type or protein as an antigenic, epitopic, or surface marker. In such instances, an antibody can be raised against the unique cell surface marker and a drug can be linked to the antibody. Upon administration of the drug/antibody complex to a patient, the binding of the antibody to the cell surface marker results in the delivery of a relatively high concentration of the drug to the diseased tissue or organ. Similar methods can be used where a particular cell type in the diseased organ expresses a unique cell surface receptor or a ligand for a particular receptor. In these cases, the drug can be linked to the specific ligand or to the receptor, respectively, thus providing a means to deliver a relatively high concentration of the drug to the diseased organ.

One of the important proteins involved in angiogenesis is Angiostatin protein. (see United States Patent No. 5,639,725 to O'Reilly et al., which is incorporated in its entirety by reference herein). Angiostatin protein preferably has a molecular weight of between approximately 38,000 Daltons and 45,000 Daltons as determined by reducing polyacrylamide gel electrophoresis, and has an amino acid sequence substantially similar to that of a plasminogen fragment beginning at approximately amino acid number 98 of an intact plasminogen molecule. Angiostatin protein has "endothelial inhibiting activity" such that it has the capability to inhibit angiogenesis in general and, for example, to inhibit the growth of bovine capillary

endothelial cells in culture in the presence of fibroblast growth factor.

Angiostatin protein may be produced from recombinant sources, from genetically altered cells implanted into animals, from tumors, and from cell cultures as well as other sources. Angiostatin protein can be isolated from body fluids including, but not limited to, serum and urine. Recombinant techniques include gene amplification from DNA sources using the polymerase chain reaction (PCR), and gene amplification from RNA sources using reverse transcriptase/PCR.

Angiostatin protein has been shown to be capable of inhibiting the growth of endothelial cells *in vitro* and *in vivo*. Angiostatin protein does not inhibit the growth of cell lines derived from other cell types. Specifically, Angiostatin protein has no effect on Lewis lung carcinoma cell lines, mink lung epithelium, 3T3 fibroblasts, bovine aortic smooth muscle cells, bovine retinal pigment epithelium, MDCk cells (canine renal epithelium), WI38 cells (human fetal lung fibroblasts), EFN cells (murine fetal fibroblasts) and LM cells (murine connective tissue). Endogenous Angiostatin protein in a tumor bearing mouse is effective at inhibiting metastases at a systemic concentration of approximately 10 mg Angiostatin protein/kg body weight.

Angiostatin protein has a specific three dimensional conformation that is defined by the kringle region of the plasminogen molecule. (Robbins, K.C., "The plasminogen-plasmin enzyme system" Hemostasis and Thrombosis, Basic Principles and Practice, 2nd Edition, ed. by Colman, R.W. et al. J.B. Lippincott Company, pp. 340-357, 1987). There are five such kringle regions, which are conformationally related motifs and have substantial sequence homology in the amino terminal portion of the plasminogen molecule. See Figure 1 for a schematic diagram of the structure of the plasminogen molecule.

The amino acid sequence of the complete murine plasminogen molecule is shown in SEQ ID NO:81.

A preferred amino acid sequence for human Angiostatin protein is shown in Figure 2.

As used herein, "kringle 1" means a protein derivative of plasminogen having an endothelial cell inhibiting activity, and having an amino acid sequence comprising a sequence homologous to kringle 1, exemplified by, but not limited to that of human kringle 1 corresponding to amino acid positions 11 to 90 (inclusive) of Angiostatin protein of SEQ ID NO:1. As used herein, "kringle 2" is exemplified by, but not limited, to amino acid positions 94 to 172 (inclusive) of Angiostatin protein of SEQ ID NO:1. As used herein, "kringle 3" is exemplified by, but not limited to, amino acid positions 185 to 263 (inclusive) of Angiostatin protein of SEQ ID NO:1. As used herein, "kringle 4" is exemplified by, but not limited to, amino acid positions 288 to 366 (inclusive) of Angiostatin protein of SEQ ID NO:1.

Furthermore, it is understood that a variety of silent amino acid substitutions, additions, or deletions can be made in the above identified kringle fragments, which do not significantly alter the fragments' endothelial cell inhibiting activity, and which are, therefore, not intended to exceed the scope of the claims.

Each kringle region of the plasminogen molecule contains approximately 80 amino acids and contains 3 disulfide bonds. Anti-angiogenic Angiostatin protein may contain a varying amount of amino- or carboxy-terminal amino acids from the interkringle regions and may have some or all of the naturally occurring di-sulfide bonds reduced. Angiostatin protein may also be provided in an aggregate, non-refolded, recombinant form. Additionally, individual and groups of kringle peptides may be useful for inhibition of angiogenesis (see PCT/US96/05856, which is incorporated herein by reference).

The cDNA sequence for human Angiostatin protein is provided as SEQ ID NO: 29.

It is contemplated that any isolated protein or peptide having a three dimensional kringle-like conformation or cysteine motif that has anti-angiogenic activity *in vivo*, is also an Angiostatin protein compound. The amino acid sequence of the Angiostatin protein of the present invention may vary depending upon, for example, from which species the plasminogen molecule is derived. Thus, although the Angiostatin protein of the present invention that is derived from human plasminogen has a slightly

different sequence than Angiostatin protein derived from mouse. it has anti-angiogenic activity as shown in a mouse tumor model.

Another important angiogenesis-related protein is Endostatin protein. (see Unites States Patent Application Serial No. 08/740.168 and WO 97/15666 O'Reilly et al.. both of which are incorporated in their entirety by reference herein) Endostatin protein is a potent and specific inhibitor of endothelial proliferation and angiogenesis. Systemic therapy with Endostatin protein causes a nearly complete suppression of tumor induced angiogenesis.

Endostatin protein has a molecular weight of approximately 18,000 to approximately 20,000 Daltons as determined by non-reduced and reduced gel electrophoresis, respectively. and is capable of inhibiting endothelial cell proliferation in cultured endothelial cells. Endostatin protein has an amino acid sequence substantially similar to a fragment of a collagen molecule and whereas it binds to a heparin affinity column, it does not bind to a lysine affinity column.

Endostatin protein can be isolated from murine hemangioendothelioma EOMA. Endostatin protein may also be produced from recombinant sources, from genetically altered cells implanted into animals, from tumors, and from cell cultures as well as other sources. Endostatin protein can be isolated from body fluids including, but not limited to. serum and urine. Recombinant techniques include gene amplification from DNA sources using the polymerase chain reaction (PCR), and gene amplification from RNA sources using reverse transcriptase/PCR.

Alternatively, endothelial proliferation inhibiting proteins, or Endostatin proteins, of the present invention may be isolated from larger known proteins, such as human alpha 1 type XVIII collagen and mouse alpha 1 type XVIII collagen, proteins that share a common or similar N-terminal amino acid sequence. Examples of other potential Endostatin protein source materials having similar N-terminal amino acid sequences include Bos taurus pregastric esterase, human alpha 1 type XV collagen, NAD-dependent formate dehydrogenase (EC 1.2.1.2) derived from Pseudomonas sp., s11459 hexon protein of bovine adenovirus type 3. CELF21D12 2 F21d12.3 Caenorhabditis

elegans gene product. VAL1 TGMV AL1 protein derived from tomato golden mosaic virus. s01730 hexon protein derived from human adenovirus 12, and Saccharomyces cerevisiae.

Human Endostatin can be further characterized by its preferred amino acid sequence as set forth in Figure 3 and in SEQ ID NO: 2. The preferred sequence of the first 20 amino-terminal amino acids corresponds to a C-terminal fragment of collagen type XVIII or collagen type XV. Specifically, in one embodiment the amino terminal amino acid sequence of Endostatin protein corresponds to an internal 20 amino acid peptide region found in mouse collagen alpha 1 type XVIII starting at amino acid 1105 and ending at amino acid 1124. The amino terminal amino acid sequence of the inhibitor also corresponds to an internal 20 amino acid peptide region found in human collagen alpha 1 type XVIII starting at amino acid 1132 and ending at amino acid 1151. The cDNA sequence for Endostatin protein is provided as SEQ ID NO: 30.

Both Angiostatin protein and Endostatin protein specifically and reversibly inhibit endothelial cell proliferation and may be used, for example, as a birth control drug, for treating angiogenesis-related diseases, particularly angiogenesis-dependent cancers and tumors, and for curing angiogenesis-dependent cancers and tumors. Therapies directed at control of the angiogenic processes could lead to the abrogation or mitigation of such diseases mediated by angiogenesis. Potential therapies useful for controlling angiogenic processes may involve recognition of antigenic markers and receptors associated with angiogenesis and subsequent modification of such markers and receptors. For example, once a receptor for an angiogenesis-related protein is identified, it can be blocked, thereby inhibiting the effect of the angiogenesis-related protein and ultimately reducing angiogenesis.

One technique that is useful for identifying antigenic markers and receptors is phage-display technology. (see for example Phage Display of Peptides and Proteins: A Laboratory Manual. Edited by Brian K. Kay et al. Academic Press San Diego, 1996) Phage-display technology is a powerful tool for the identification of individual epitopes that interact with ligands such

as proteins and antibodies. Phage peptide libraries typically comprise numerous different phage clones, each expressing a different peptide, encoded in a single-stranded DNA genome as an insert in one of the coat proteins. In an ideal phage library the number of individual clones would be 20^n, where "n" equals the number of residues that make up the random peptides encoded by the phage. For example, if a phage library was screened for a seven residue peptide, the library in theory would contain 20^7(or 1.28×10^9) possible 7-residue sequences. Therefore, a 7-mer peptide library should contain approximately 10^9 individual phage.

Phage clones displaying peptides that are able to mimic epitopes recognized by a particular protein (or antibody), are selected from the library based upon their binding affinity to that protein (or antibody), and the sequences of the inserted peptides are deduced from the DNA sequences of the phage clones. This approach is particularly desirable because no prior knowledge of the primary sequence of the target protein is necessary, epitopes represented within the target, either by a linear sequence of amino acids (linear epitope) or by the spatial juxtaposition of amino acids distant from each other within the primary sequence (conformational epitope) are both identifiable, and peptidic mimotopes of epitopes derived from non-proteinaceous molecules such as lipids and carbohydrate moieties can also be generated.

With regard to angiogenesis-related disease, it is evident that angiogenesis-related proteins such as Angiostatin protein and Endostatin protein play an important role in the development of disorders such as cancer. What is needed therefore, is the development of methods and compositions for the identification of receptors and molecules that bind such proteins. The identification of such receptors and molecules would facilitate the understanding of angiogenesis-related protein influence and interaction, and consequently enable the development of drugs to modify the activity of these proteins as necessary.

SUMMARY OF THE INVENTION

In accordance with the present invention, compositions and methods are provided that are effective for modulating angiogenesis, and inhibiting all forms of unwanted angiogenesis, especially angiogenesis related to tumor growth. Specifically, the present invention includes peptides and proteins that bind to angiogenesis-related peptides and proteins, such as Angiostatin protein or Endostatin protein. Identification of these binding molecules and proteins has improved the understanding of angiogenesis-related protein influence, and has also made possible the development of therapeutic agents for modifying angiogenesis associated with disorders such as cancer and tumor development.

Angiostatin protein and Endostatin protein are defined by their ability to inhibit the angiogenic activity of endogenous growth factors such as bFGF on endothelial cells, *in vitro* and tumor growth *in vivo*. Angiostatin protein contains approximately kringle regions 1 through 4 of plasminogen and is a protein having a molecular weight of between approximately 38,000 Daltons and 45,000 Daltons as determined by reducing polyacrylamide gel electrophoresis. In a preferred embodiment, Angiostatin protein has an amino acid sequence substantially similar to that of a fragment of murine plasminogen beginning at amino acid number 98.

Endostatin protein is a collagen fragment, and is a protein having a molecular weight of between approximately 18,000 Daltons and 20,000 Daltons as determined by non-reducing and reducing gel electrophoresis, respectively. Endostatin protein is further characterized by its ability to bind a heparin affinity column and inability to bind a lysine affinity column. A preferred sequence of the first 20 N-terminal amino acids of Endostatin protein corresponds to a C-terminal fragment of collagen type XVIII or XV.

The present invention also encompasses nucleotide sequences encoding peptides and proteins that bind angiogenesis-related peptides and proteins, as well as expression vectors containing nucleotide sequences encoding such binding peptides and proteins, and cells containing one or more expression vectors containing nucleotide sequences encoding such peptides and

proteins. The present invention further encompasses gene therapy methods whereby nucleotide sequences encoding angiogenesis-related protein binding peptides and proteins are introduced into a patient to modify *in vivo* Angiostatin protein or Endostatin protein levels.

The present invention also includes diagnostic methods and kits for detection and measurement of peptides and proteins that bind angiogenesis-related proteins in biological fluids and tissues, and for localization of such peptides and proteins in tissues and cells. The diagnostic method and kit can be in any configuration well known to those of ordinary skill in the art.

The present invention includes peptides and proteins that bind Angiostatin protein or Endostatin protein and cause the transmission of an appropriate signal to a cell and act as agonists or antagonists of angiogenesis.

In addition, the present invention includes fragments of proteins that bind angiogenesis-related proteins, and analogs thereof, that can be labeled isotopically, or with other molecules or proteins, for use in the detection and visualization of angiogenesis-related protein binding sites with techniques, including, but not limited to, positron emission tomography, autoradiography, flow cytometry, radioreceptor binding assays, and immunohistochemistry.

The peptides and analogs of the present invention also act as agonists and antagonists for Angiostatin protein or Endostatin protein receptors, thereby enhancing or blocking the biological activity of Angiostatin protein or Endostatin protein. Such peptides and proteins are used in the isolation of Angiostatin protein or Endostatin protein receptors.

The present invention includes molecular probes for the ribonucleic acid and deoxyribonucleic acid involved in transcription and translation of angiogenesis-related protein binding peptides and proteins. These molecular probes provide means to detect and measure angiogenesis-related protein biosynthesis in tissues and cells.

Accordingly, it is an object of the present invention to provide compositions and methods comprising peptides and proteins that bind angiogenesis-related peptides and proteins.

It is another object of the present invention to provide compositions and methods for treating diseases and processes that are mediated by angiogenesis.

It is yet another object of the present invention to provide diagnostic or prognostic methods and kits for detecting the presence and amount of angiogenesis-related protein binding peptides in a body fluid or tissue.

It is yet another object of the present invention to provide compositions and methods for treating diseases and processes that are mediated by angiogenesis including, but not limited to, hemangioma, solid tumors, blood borne tumors, leukemia, metastasis, telangiectasia, psoriasis, scleroderma, pyogenic granuloma, myocardial angiogenesis, Crohn's disease, plaque neovascularization, coronary collaterals, cerebral collaterals, arteriovenous malformations, ischemic limb angiogenesis, corneal diseases, rubeosis, neovascular glaucoma, diabetic retinopathy, retrolental fibroplasia, arthritis, diabetic neovascularization, macular degeneration, wound healing, peptic ulcer, Helicobacter related diseases, fractures, keloids, vasculogenesis, hematopoiesis, ovulation, menstruation, placentation, and cat scratch fever.

It is another object of the present invention to provide compositions and methods for treating or repressing the growth of a cancer.

Another object of the present invention to provide compositions and methods for the detection or prognosis of cancer.

It is another object of the present invention to provide compositions and methods for use in visualizing and quantitating sites of Angiostatin protein or Endostatin protein binding *in vivo* and *in vitro*.

It is yet another object of the present invention to provide compositions and methods for use in detection and quantification of Angiostatin protein or Endostatin protein biosynthesis.

Another object of the present invention to provide receptors that bind angiogenesis-related proteins, such as Angiostatin protein or Endostatin protein.

Yet another object of the present invention is to identify proteins, and fragments thereof, that interact and regulate the activity of angiogenesis-related proteins such as Angiostatin protein or Endostatin protein.

Still another object of the present invention is to provide proteins, and fragments thereof, that are involved in the transport of angiogenesis-related proteins such as Angiostatin protein or Endostatin protein.

Another object of the present invention is to provide proteins, and fragments thereof that function as substrates through which angiogenesis-related proteins exert their activities.

It is yet another object of the present invention to provide a therapy for cancer that has minimal side effects.

Yet another object of the present invention is to provide methods and compositions comprising proteins and peptides, that bind Angiostatin protein comprising laminin protein, and/or proteins and peptides that bind Endostatin protein comprising tropomyosin.

Still another object of the present invention is to provide methods and compositions comprising peptides, that bind Angiostatin protein and/or Endostatin protein, linked to a cytotoxic agent for treating or repressing the growth of a cancer.

It is another object of the present invention to provide fusion proteins comprising laminin and tropomyosin for binding and regulating Angiostatin protein and/or Endostatin protein.

Yet another object of the present invention is to provide fusion proteins comprising peptides or proteins that bind Angiostatin protein and/or Endostatin protein.

Another object of the present invention is to provide methods and compositions for targeted delivery of angiogenesis-related protein compositions to specific locations.

Yet another object of the invention is to provide compositions and methods useful for gene therapy for the modulation of angiogenic processes.

These and other objects, features and advantages of the present invention will become apparent after a review of the following detailed description of the disclosed embodiments and the appended claims.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a schematic representation of the structure of human plasminogen and its kringle fragments. Human plasminogen is a single chain protein containing 791 amino acids with one site of N-linked glycosylation at Asn^{289}. The non-protease region of human plasminogen consists of the N-terminal 561 amino acids existing in five separate domains, termed kringles as shown in circles (K1, K2, K3, K4 and K5), along with the protein sequences (or with the amino acids) that separate these structures. Each triple disulfide bonded kringle contains approximately 80 amino acids. Angiostatin covers the first 4 of these kringle domains (K1-4), kringle 3 (K1-3) and kringle 4 (K4) are obtained by digestion of human plasminogen with elastase. The rest of the kringle fragments are recombinant proteins expressed in *E. coli*. 'SS' signifies signal sequence. 'PA' signifies preactivation protein.

Figure 2 shows the preferred amino acid sequence of human Angiostatin protein.

Figure 3 shows the preferred amino acid sequence of human Endostatin protein.

Figure 4 provides graphs demonstrating binding preference of linear peptides (selected by display technology) for Angiostatin protein over plasminogen.

Figure 5 provides graphs demonstrating binding preference of cyclized peptides (selected by display technology) for Angiostatin protein over plasminogen.

Figure 6A provides the complete amino acid sequence of laminin protein (SEQ ID NO: 113), and Figure 6B provides the complete nucleotide sequence of laminin protein (SEQ ID: 114).

Figure 7 shows the three motifs of laminin protein corresponding to Angiostatin protein selected peptides in a single 60 amino acid long region.

Figure 8 is a graph showing the results of an ELISA assay demonstrating the interaction of tropomyosin and Endostatin protein.

Figure 9 provides the results of an *in vivo* experiment for assessing the interaction of peptide E37 and Endostatin protein.

Figure 10 provides a graph showing the results of an *in vivo* experiment for assessing the interaction of peptide E37 and Endostatin protein as the mean, standard deviation, T/C and the results of a 2-tailed T-test.

DETAILED DESCRIPTION

The following description includes the best presently contemplated mode of carrying out the invention. This description is made for the purpose of illustrating the general principles of the inventions and should not be taken in a limiting sense. All references listed or cited herein are incorporated by reference in their entirety.

As used herein, the term "Angiostatin protein" refers to a protein containing approximately kringle regions 1 through 4 of a plasminogen molecule and having a molecular weight of between approximately 38,000 Daltons and 45,000 Daltons as determined by reducing polyacrylamide gel electrophoresis. Angiostatin protein preferably has an amino acid sequence substantially similar to that of a fragment of murine plasminogen beginning at approximately amino acid number 98. The description of Angiostatin protein is provided in United States Patent No. 5,639,725 to O'Reilly et al. A preferred amino acid sequence for human Angiostatin protein is shown in Figure 3.

As used herein, the term "Endostatin protein" refers to a protein that is a collagen molecule fragment and has a molecular weight of between approximately 18,000 Daltons and 20,000 Daltons as determined by non-reducing and reducing electrophoresis. respectively. The preferred sequence of the first 20 N-terminal amino acids of Endostatin protein corresponds to a C-terminal fragment of collagen type XVIII or XV. The description of Endostatin protein is provided in Unites States

Patent Application Serial No. 08/740.168 and WO 97/15666 O'Reilly et al. A preferred amino acid sequence for human Endostatin protein is shown in Figure 3.

As used herein, the term "angiogenesis-related protein" refers to Angiostatin protein and Endostatin protein, and active fragments and homologs thereof, involved in blood vessel growth and development.

The term "angiogenesis-related protein" includes proteins that are animal or human in origin and also includes proteins that are made synthetically by chemical reaction, or by recombinant technology in conjunction with expression systems.

As used herein, the term "binding peptide" refers to peptides, active fragments and homologs thereof, that bind angiogenesis-related proteins. It will be understood by those skilled in the art that the preferred binding peptides include peptide analogs, which are defined herein as peptides capable of binding angiogenesis-related proteins. Exemplary binding peptides, their amino acid sequences (SEQ ID NOS: 3-28, SEQ ID NOS: 31-42 and 82-113), and their experimental abbreviations are set forth in the description below. The binding peptides and proteins herein are other than naturally occurring immunoglobulin antibody molecules.

The terms "a", "an" and "the" as used herein are defined to mean "one or more" and include the plural unless the context is inappropriate. As employed herein, the phrase "biological activity" refers to the functionality, reactivity, and specificity of compounds that are derived from biological systems or those compounds that are reactive to them, or other compounds that mimic the functionality, reactivity, and specificity of these compounds. Examples of suitable biologically active compounds include enzymes, antibodies, antigens and proteins.

The term "bodily fluid," as used herein, includes, but is not limited to, saliva, gingival secretions, cerebrospinal fluid, gastrointestinal fluid, mucous, urogenital secretions, synovial fluid, blood, serum, plasma, urine, cystic fluid, lymph fluid, ascites, pleural effusion, interstitial fluid, intracellular fluid, ocular fluids, seminal fluid, mammary secretions, and vitreal fluid, and nasal secretions.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one having ordinary skill in the art to which this invention belongs. Although other materials and methods similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described.

The present invention includes methods and compositions for the detection and treatment of diseases and processes that are mediated by or associated with angiogenesis. The compositions comprise peptides, proteins, active fragments, and homologs thereof, that are capable of binding to angiogenesis-related proteins. In particular, the peptides and proteins of the present invention are capable of interacting with or binding to Angiostatin protein and/or Endostatin protein.

The present invention includes the use of peptides and proteins such as receptors, that bind angiogenesis-related proteins for detection of angiogenesis-related proteins, such as Angiostatin protein and Endostatin protein, in body fluids and tissues for the purpose of diagnosis or prognosis of diseases such as cancer. In addition, the present invention also includes the detection of Angiostatin protein and Endostatin protein binding sites and receptors in cells and tissues. The present invention further includes methods of treating or preventing angiogenic diseases and processes including, but not limited to, arthritis and tumors by using angiogenesis protein-binding peptides and proteins for altering the production, administration and activity of Angiostatin protein or Endostatin protein. It is to be understood that the proteins and peptides that bind angiogenesis-related proteins such as Angiostatin protein or Endostatin protein can be animal or human in origin. Such binding peptides can also be produced synthetically by chemical reaction or by recombinant techniques in conjunction with expression systems.

Angiogenesis-related proteins can be isolated from body fluids including, but not limited to, serum, urine and ascites, or synthesized by chemical or biological methods (including cell culture, recombinant gene expression, peptide synthesis). The proteins may also be obtained by *in vitro* enzymatic catalysis of

plasminogen or plasmin to yield active Angiostatin protein, or of collagen to yield active Endostatin protein. Amino acid sequences of preferred peptides and proteins that bind Angiostatin protein or Endostatin protein are provided as SEQ ID NOS: 3-28, 31-42 and 82-113. Recombinant techniques include gene amplification from DNA sources using the polymerase chain reaction (PCR), and gene amplification from RNA sources using reverse transcriptase/PCR. General references for methods that can be used to perform the various PCR and cloning procedures described herein can be found in Molecular Cloning: A Laboratory Manual (Sambrook et al., eds. Cold Spring Harbor Lab Publ. 1989, latest edition), which is hereby incorporated by reference. Both Angiostatin protein and Endostatin protein inhibit the growth of blood vessels into tissues such as unvascularized or vascularized tumors.

The present invention further encompasses compositions and methods comprising, vectors containing nucleotide sequences encoding peptides and proteins capable of binding angiogenesis-related proteins comprising Angiostatin protein or Endostatin protein, wherein the vector is capable of expressing such binding peptides when present in a cell, a composition comprising a cell containing such a vector. Nucleotide sequences of preferred peptides and proteins that bind Angiostatin protein or Endostatin protein are provided as SEQ ID NOS: 43-80. Because of degeneracy in the genetic code, alternative nucleotide sequences can code for a peptide with the same sequence. The present invention further includes a method comprising, implanting into a human or non-human animal, a cell containing such a vector.

The present invention also encompasses gene therapy whereby genes encoding peptides that bind angiogenesis-related proteins, such as Angiostatin protein or Endostatin protein, are regulated in a patient. Various methods of transferring or delivering DNA to cells for expression of the gene product protein, otherwise referred to as gene therapy, are disclosed in Gene Transfer into Mammalian Somatic Cells *in vivo*, N. Yang, Crit. Rev. Biotechn. 12(4): 335-356 (1992), which is hereby incorporated by reference. Gene therapy encompasses

incorporation of DNA sequences into somatic cells or germ line cells for use in either *ex vivo* or *in vivo* therapy. Gene therapy functions to replace genes, augment normal or abnormal gene function, and to combat infectious diseases and other pathologies.

Methods for treating medical problems with gene therapy include therapeutic strategies such as identifying the defective gene and then adding a functional gene to either replace the function of the defective gene or to augment a slightly functional gene: or prophylactic strategies, such as adding a gene for the product protein that will treat the condition or that will make the tissue or organ more susceptible to a treatment regimen. As an example of a prophylactic strategy, a gene for a peptide or protein comprising for example, laminin protein that binds to Angiostatin protein, and/or tropomyosin that binds Endostatin, may be placed in a patient to modify the occurrence of angiogenesis.

Many protocols for transfer of peptide DNA or peptide regulatory sequences are envisioned in this invention. Examples of such technology is found in Transkaryotic Therapies, Inc., of Cambridge, Massachusetts, using homologous recombination to insert a "genetic switch" that turns on an erythropoietin gene in cells. See Genetic Engineering News, April 15, 1994. Such "genetic switches" could be used to activate the desired peptide in cells not normally expressing the corresponding gene.

Gene transfer methods for gene therapy fall into three broad categories-physical (e.g., electroporation, direct gene transfer and particle bombardment), chemical (lipid-based carriers, or other non-viral vectors) and biological (virus-derived vector and receptor uptake). Gene therapy methodologies can also be described by delivery site. Fundamental ways to deliver genes are familiar to those skilled in the art and include *ex vivo* gene transfer, *in vivo* gene transfer, and *in vitro* gene transfer.

Chemical methods of gene therapy may involve a lipid based compound (such as lipofectins or cytofectins), not necessarily a liposome, to ferry the DNA across the cell membrane. Another chemical method may use receptor-based endocytosis, which involves binding a specific ligand to a cell

surface receptor and enveloping and transporting it across the cell membrane. The ligand binds to the DNA and the whole complex is transported into the cell. The ligand gene complex is injected into the blood stream and then target cells that have the receptor will specifically bind the ligand and transport the ligand-DNA complex into the cell.

Many gene therapy methodologies employ viral vectors to insert genes into cells. For example, altered retrovirus vectors have been used in *ex vivo* methods to introduce genes into peripheral and tumor-infiltrating lymphocytes, hepatocytes, epidermal cells, myocytes, or other somatic cells. These altered cells are then introduced into the patient to provide the gene product from the inserted DNA.

Viral vectors have also been used to insert genes into cells using *in vivo* protocols. To direct tissue-specific expression of foreign genes, cis-acting regulatory elements or promoters that are known to be tissue specific can be used. Alternatively, this can be achieved using *in situ* delivery of DNA or viral vectors to specific anatomical sites *in vivo*. A viral vector can be delivered directly to the *in vivo* site, by a catheter for example, thus allowing only certain areas to be infected by the virus, and providing long-term, site specific gene expression. *In vivo* gene transfer using retrovirus vectors has also been demonstrated in mammary tissue and hepatic tissue by injection of the altered virus into blood vessels leading to the organs.

Fundamental advantages of retroviral vectors for gene transfer include efficient infection and gene expression in most cell types, precise single copy vector integration into target cell chromosomal DNA, and ease of manipulation of the retroviral genome.

According to the methods used herein, a library of phage displaying potential binding peptides was incubated with immobilized angiogenesis-related proteins to select clones encoding recombinant peptides that specifically bound the immobilized angiogenesis-related protein. The phage that encoded the recombinant peptides were amplified after three rounds of biopanning (binding to the immobilized angiogenesis-related proteins) and individual viral plaques, each expressing a

different recombinant protein, or binding peptide, were then expanded to produce amounts of peptides sufficient to perform a binding assay.

One possible technique for identifying the angiogenesis-related binding peptides involves the method wherein the DNA encoding recombinant binding peptides can be subsequently modified for ligation into a eukaryotic protein expression vector. This modification entails removal of the 5' secretion signal sequence and the addition of a translation initiation codon that direct translation of the recombinant binding peptide. Stop codons are also incorporated into the construct in a region downstream of the coding sequence. All cloning procedures employed in the development of the present invention were carried out according to standard laboratory practice.

Methods for preparing libraries containing diverse population of various types of molecules such as peptides, polypeptides, protein, and fragments thereof are known in the art and are commercially available (see, for example, Ecker and Crooke, Biotechnology 13:351-360 (1995), and the references cited therein).

The phage display libraries used in for present invention include the Ph.D.-7 phage display library (New England BioLabs Cat #8100), a combinatorial library consisting of random peptide 7-mers. The Ph.D.-7 phage display library consists of linear 7-mer peptides fused to the pIII coat protein of M13 via a Gly-Gly-Gly-Ser flexible linker. The library contains 2.8 x 10^9 independent clones and is useful for identifying targets requiring binding elements concentrated in a short stretch of amino acids.

Another phage display library used for the present invention includes the Ph.D.-C7C library (New England BioLabs Cat # 8120) which is a combinatorial library consisting of random peptide 7-mers flanked by cysteine residues that under non-denaturing conditions are disulfide bonded resulting in the display of cyclized peptides. In non-reducing conditions, the cysteines form a disulfide bond resulting in each peptide being constrained in a disulfide loop. The library contains 3.7 x 10^9 independent clones that like the Ph.D.-7 library the peptides are fused to the pIII coat protein of M13 via a Gly-Gly-Gly-Ser flexible linker.

Constrained libraries are useful in the identification of structural epitopes. This library was screened for the binding of 7 amino acid cyclized peptides.

Phage selection was conducted according to methods known in the art and according to manufacturers' recommendations. The "target" proteins, recombinant human Angiostatin protein, or recombinant human Endostatin protein, were coated overnight onto tissue culture plates in humidified containers. In the first round of panning approximately $2x10^{11}$ phage were incubated on the protein coated plate for 60 minutes at room temperature while rocking gently. The plates were then washed several times using standard wash solutions such as TBS (50mM Tris-HCl (pH 7.5), 150 mM NaCl) containing 0.1% Tween 20. The binding phage were then collected and amplified following elution using the target protein. Secondary and tertiary pannings were performed as necessary.

Following the last screening individual colonies of phage-infected bacteria were picked at random, the phage DNA was isolated and subjected to automated dideoxy sequencing. The sequence of the displayed peptides were deduced from the DNA sequence.

Use of phage-display technology is particularly desirable for the detection of molecules that function as receptors for angiogenesis-related proteins. For example, a specific receptor may be displayed on the surface of the phage such that it may bind its ligand. The receptor can then be modified by, for example, *in vitro* mutagenesis and variants having higher binding affinity for the ligand selected. As used herein, the term "receptor" means molecule that binds a specific, or group of specific angiogenesis-related proteins. The natural "receptors" could be expressed on the surface of a population of cells, or they could be the extracellular domain of such a molecule (whether such a form exists naturally or not), or a soluble molecule performing natural binding function in the plasma, or within a cell or organ.

Alternatively, the phage-receptor can be used as the basis of a rapid screening system for the binding of ligands such as Angiostatin protein and Endostatin protein, altered ligands, or

potential drug candidates. The advantages of this system namely, of simple cloning, convenient expression, standard reagents and easy handling makes the drug screening application particularly attractive.

In addition, the phage-receptor may be used to identify binding peptides and proteins for better understanding, and ultimately modifying, the role of angiogenesis-related proteins in angiogenesis, and in the manifestation of angiogenesis-related disease. For example, the binding peptides may be used to identify proteins that interact with, and/or regulate (either positively or negatively), the activity of angiogenesis-related proteins such as Angiostatin protein or Endostatin protein. In addition, such binding peptides may also be used to identify other proteins and molecules involved in the transport of angiogenesis-related proteins, and substrates through which angiogenesis-related proteins exert their activities.

Following synthesis of the peptides and proteins that bind angiogenesis-related proteins, the peptides and proteins may be added to the *in vitro* assays to further determine the biological activity of Angiostatin protein or Endostatin protein. These assays are familiar to those skilled in the art and include HUVEC and BCE proliferation assays, HUVEC wound/migration assay, endothelial cell tube forming assay, CAM assay, Matrigel invasion assay and the rat aortic assay. Specifically, the peptides and proteins that inhibit or stimulate the activity of Angiostatin protein or Endostatin protein are identified. The ability of a peptide or protein to inhibit or stimulate the activity of either Angiostatin protein or Endostatin protein in these assays would indicate that the peptide or protein is able to mimic the interaction of Angiostatin protein or Endostatin protein with proteins that regulate their activity.

The biological activity of the binding peptides and proteins may also be tested *in vivo*. The peptides or proteins may be pre-incubated with their target angiogenesis-related protein (Angiostatin protein or Endostatin protein) prior to being used in either the B16Bl6 metastasis assay or the Lewis Lung Carcinoma primary tumor or metastasis assays. In such experiments a comparison would be made between the activity of the target

protein Angiostatin protein or Endostatin protein and the target protein Angiostatin protein or Endostatin protein pre-bound with the peptide or protein. If the peptide/target protein interactions mimic important biological interactions involved in the activity of Angiostatin protein or Endostatin protein then it would be expected that the anti-angiogenic activity of the target protein would be neutralized by the binding of the peptide or protein.

A complementary approach to determining whether the peptides or proteins mimic the epitopes of proteins that interact with Angiostatin protein or Endostatin protein would be to use the synthesized peptide and protein to generate antibodies. These anti-peptide and anti-protein antibodies would recognize proteins with which Angiostatin protein or Endostatin protein interact. By binding to sites important for the interaction of Angiostatin protein or Endostatin protein with their binding proteins these antibodies would effect the anti-angiogenic activity of the Angiostatin protein or Endostatin protein. Thus, these anti-peptide and anti-protein antibodies can be assayed for their ability to affect the activity of Angiostatin protein or Endostatin protein.

The anti-peptide and anti-protein antibodies may also be used to screen phage expression libraries such as a λgt11 expression library. Such an approach would enable the cloning of the cDNA corresponding to proteins that interact with Angiostatin protein or Endostatin protein. Once the cDNAs have been identified they may be produced using recombinant technology and their anti-angiogenic activities in various assays determined alone and in combination with their target angiogenesis-related protein.

Using the methods described above and further described below in the Examples, the peptides in Tables 1-4 have been identified as linear or cyclized peptides that bind Angiostatin protein or Endostatin protein.

Two phage-display libraries were screened, the first was a linear library that encoded for linear 7 residue peptides, and the second was a disulfide constrained peptide library. Specifically, the second library consisted of 7-mer randomized peptide sequences which were flanked by a pair of cysteine

residues. The cysteine residues spontaneously form disulfide cross-links, resulting in the phage displaying cyclized peptides.

Table 1
Linear Peptides Binding Angiostatin Protein

Peptide	Amino Acid Sequence	SEQ ID NO:
A1	E R V N D D T G G G S	SEQ ID NO:3
A3	D R S G A I K G G G S	SEQ ID NO:4
A7	L D R A N V F G G G S	SEQ ID NO:5
A9	S P L G G S E G G G S	SEQ ID NO:6
A10	H A I Y P R H G G G S	SEQ ID NO:7

Table 2
Cyclized Peptides Binding Angiostatin Protein

Peptide	Amino Acid Sequence	SEQ ID NO:
A25	C W S Y E W S K C G G G	SEQ ID NO:8
A31	C W S L E Q S K C G G G	SEQ ID NO:9
A35	C W S L E W Q K C G G G	SEQ ID NO:10
A28	C W S L E T T K C G G G	SEQ ID NO:11
A33	C W S L E H Q K C G G G	SEQ ID NO:12
A34	C W S L E I L K C G G G	SEQ ID NO:13
A30	C W T L E S T K C G G G	SEQ ID NO:14
A32	C G D M S D R P C G G G	SEQ ID NO:15

Table 3
Linear Peptides Binding Endostatin Protein

Peptide	Amino Acid Sequence	SEQ ID NO:
E15	H K R P R N N G G G S	SEQ ID NO:16
E12	T K H R A G R G G G S	SEQ ID NO:17
E13	W H R S V W K G G G S	SEQ ID NO:18
E14	S P Q P F E E G G G S	SEQ ID NO:19
E16	F T E P T H K G G G S	SEQ ID NO:20
E17	K D Y A L P P G G D S	SEQ ID NO:21
E18	S K I A P I M G G G S	SEQ ID NO:22
E20	W R Q T R K D G G G S	SEQ ID NO:23
E22	G K P M P P M G G G S	SEQ ID NO:24

Table 4
Cyclized Peptides Binding Endostatin Protein

Peptide	Amino Acid Sequence	SEQ ID NO:
E37	C T H W W H K R C G G G S	SEQ ID NO:25
E41	C S L T P H R Q C G G G S	SEQ ID NO:26
E45	C E K E K P M T C G G G S	SEQ ID NO:27
E48	C A P P G L A R C G G G S	SEQ ID NO:28

Table 5
Linear Peptides that Preferentially Bind to
Angiostatin protein over Plasminogen

Peptide	Amino Acid Sequence	SEQ ID NO:
PAL-49	K C C Y Y A K G G G S	SEQ ID NO:31
PAL-51	K C C Y P S A G G G S	SEQ ID NO:32
PAL-54	R Q P P H L H G R G S	SEQ ID NO:33
PAL-56	H K Y I S A T G G G S	SEQ ID NO:34
PAL-66	G T L Q V L S G G G S	SEQ ID NO:35
PAL-69	K C C Y S V G G G G S	SEQ ID NO:36
PAL-70	M S Y Q W S H G G G S	SEQ ID NO:37

Table 6
Cyclized Peptides that Preferentially Bind to
Angiostatin protein over Plasminogen

Peptide	Amino Acid Sequence	SEQ ID NO:
PAC-77	C W S L E H S K C G G G S	SEQ ID NO:38
PAC-78	C V H S I E R E C G G G S	SEQ ID NO:39
PAC-82	C Y T L P P K L C G G G S	SEQ ID NO:40
PAC-88	C W S Y E W S K C G G G S	SEQ ID NO:41
PAC-91	C W S L E W Q K C G G G S	SEQ ID NO:42

Nucleic acid sequences corresponding to the above amino acid sequences are provided as SEQ ID NOS: 43-80.

The present invention encompasses the peptides set forth in Tables 5 and 6 which preferentially bind to Angiostatin protein as opposed to plasminogen. The preferential binding activity of the peptides illustrate that a preferred Angiostatin protein receptor is one of these peptides.

In addition, the present invention is related to proteins which share homologous sequences to the claimed peptides. Such proteins include human possible global

transcription activator, phosphopentomutase, ribonuclease RH precursor, soybean early nodulin, JNK activating kinase 1, IL-12 beta chain, glutathione reductase, soy bean trypsin inhibitor (kunitz), fibroblast growth factor-6, chemotaxis protein, annexin XI, WEE 1, RAS suppressor protein 1, ATP synthase gamma chain, thioredoxin, collagenese, glycoprotein B-1 precursor, dehydroquinate dehydratase, complement component C8 beta chain, ornithine decarboxylase antizyme, adenylate cyclase, and ATP synthase, alpha chain.

The homology searches for the present invention were conducted using the FASTA sequence similarity search. The peptide sequences were queried against the SwissProt data base using the default setting (ktup 2, and BLOSUM50 for the scoring matrix). The searches were conduced using the world wide web at site http://www.fasta.genome.ad.jp/. as described by W.R. Pearson & D.J. Lipman PNAS 85:2444-2448 (1988).

Following identification of the peptides described in Tables 1-6 above, further analysis was conducted in order to determine peptides that represent potential receptors, or fragments thereof, that bind Angiostatin protein or Endostatin protein. A discussion of such peptides is provided below and in the Examples.

Proteins and Peptides that Bind Angiostatin Protein

The present invention is particularly related to methods and compositions comprising basement membranes including, but not limited to, laminin protein for modulating angiogenesis, and inhibiting forms of unwanted angiogenesis, especially angiogenesis-related to tumor growth. As demonstrated in the Examples below, as a result of sequence similarities between peptides that bind Angiostatin protein (as identified by phage display techniques) and laminin protein (specifically Example 7), laminin protein is considered to comprise a receptor, or a molecule closely related to a receptor, for Angiostatin protein. In addition, laminin protein may also be considered a protein that is involved in mediating activity of Angiostatin protein.

Based on the findings of the present invention, it is believed that Angiostatin protein interacts with the beta-1 chain of laminin protein. Laminin is a basement membrane derived noncollagenous glycoprotein found in the extracellular matrix which binds type IV collagen, glycosaminoglycan and heparin, and is involved in the promotion of cellular adhesion. Laminin protein is a heterotrimeric molecule with an alpha, beta and gamma chain.

The amino acid sequence of laminin protein is set forth in Figure 6A (SEQ ID NO: 113) and the nucleotide sequence is set forth in Figure 6B (SEQ ID NO: 114).

Proteins and Peptides that bind Endostatin protein

The present invention is particularly related to methods and compositions comprising tropomyosin for modulating angiogenesis, and inhibiting forms of unwanted angiogenesis, especially angiogenesis-related to tumor growth. As demonstrated in the Examples below (specifically Examples 5, 6, 8 and 9), tropomyosin is considered to comprise a receptor, or a molecule closely related to a receptor, for Endostatin protein. In addition, tropomyosin may also be considered a protein that is involved in mediating activity of Endostatin protein.

Though not wishing to be bound by the following theory, it is thought that the E37 peptide is a mimotope of a portion of the tropomyosin protein. Specifically, the 3 dimensional shape of the E37 peptide is similar to a (3 dimensional) region of the tropomyosin protein. Mimotopic homology may be characterized as 'shape homology'. As is known by those skilled in the art, because of 3 dimensional folding of proteins, epitopes are not always comprised of linear stretches of protein sequence. Such epitopes can be mimicked by linear sequences whose shape corresponds to shape of a non-linear epitope, such peptides are said to be minotopes. An important finding of the present invention is that the E37 peptide comprises a mimotope of the actual ligand binding site of tropomyosin. Accordingly, the findings of the present inventors suprisingly define not only the Endostatin protein receptor, but also define

the actual ligand binding domain as represented by the E37 peptide.

As further detailed in the Examples, both *in vitro* and *in vivo* experiments demonstrate that tropomyosin comprises the receptor, or a molecule closely related to the receptor for Endostatin protein. Antibodies against the E37 peptide have shown that the E37 peptide and tropomyosin share a common epitope, and it is believed that by virtue of this epitope Endostatin protein and tropomyosin are binding and that the anti-angiogenic activity of Endostatin protein is mediated via this interaction. Furthermore, in vivo experiments support this conclusion as the anti-tumor activity of Endostatin protein is significantly decreased in the presence of the E37 peptide.

Though not wishing to be bound by the following theory, further support for the binding of tropomyosin and Endostatin protein and the consequences of this interaction on angiogenesis may be derived from the effect of such binding on pericytes. After endothelial cells form new blood vessels by angiogenesis they are immature and relatively unstable (they can regress and be remodeled readily). As the blood vessels become more mature and stable they associate with pericytes, which form a barrier around the endothelial cells. At some point during this process the pericytes differentiate into fibroblasts. There is some evidence that vessels associated with pericytes cannot regress and that this may explain why anti-angiogenic compounds like Angiostatin protein and Endostatin protein target the vasculature of tumors and not the blood vessels necessary for maintenance of vital organs.

Endostatin protein results in an inhibition of proliferation of PDGF-BB or 20% fetal calf serum stimulated primary cultures of pericytes and to a lesser extent fibroblasts. More specifically, Endostatin protein results in a consistent reduction in thymidine incorporation into primary cultures of pericytes and to a lesser degree fibroblasts, that are stimulated to proliferate in response to PDGF-BB or 20% fetal calf serum. The results have been confirmed using a MTT proliferation kit.

Furthermore, Endostatin protein does not appear to effect the differentiation of pericytes to fibroblasts, although

inhibition of proliferation varies depending to the degree of differentiation along the pericyte/fibroblast differentiation pathway. Endostatin protein tends to have a greater effect on cells in the earlier stages of differentiation. While such cells constitute a small fraction of the total number, inhibiting their proliferation may have significant effects on the final total cell number. Endostatin protein's effect on cells early in the differentiation pathway results in a reduction of 70-80% of the final cell number. Endostatin protein also changes the morphology of these cells.

The isoform of tropomyosin (there are over 20 different isoforms (types) of tropomyosin expressed by mammalian cells) initially identified as binding to Endostatin protein was fibroblast tropomyosin. Though not wishing to be bound by the following theory, it is believed that tropomyosin is involved in the mechanism of action of Endostatin protein and therefore pericytes are being inhibited by virtue of the fact that they are expressing fibroblast tropomyosin. Endostatin protein does not bind human cardiac tropomyosin indicating that not all isoforms of tropomyosin bind Endostatin protein. Consequently the cell-type specificity of Endostatin protein may be explained by the isoforms of tropomyosin that it expresses. We also have evidence that Endostatin protein will bind to the surface of fibroblasts.

Though not wishing to be bound by the following theory, it is believed that as a result of the the interaction between Endostatin protein and tropomyosin, Endostatin protein inhibits pericyte differentiation and channels these cells into the apoptotic pathway.

This invention is further illustrated by the following examples, which are not to be construed in any way as imposing limitations upon the scope thereof. On the contrary, it is to be clearly understood that resort may be had to various other embodiments, modifications, and equivalents thereof which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the present invention and/or the scope of the appended claims.

EXAMPLES

EXAMPLE 1

Identification of Angiogenesis-Related Binding Peptides

A "phage-display library" is a protein expression library, constructed in a vector that expresses a collection of cloned protein sequences as fusions with a phage coat protein. Thus, in the context of the present invention, single-chain recombinant proteins having the potential ability to bind angiogenesis-related proteins, are expressed as fusion proteins on the exterior of the phage particle. This "placement" advantageously allows contact and binding between the recombinant binding protein and an immobilized protein such as Angiostatin protein or Endostatin protein. Phage that bind an angiogenesis-related protein can be recovered; individual phage can then be cloned and the peptide expressed by cloned phage can be determined. Phage clones expressing binding peptides specific for angiogenesis-related proteins can be substantially enriched by serial rounds of phage binding to the immobilized protein and amplification by growth in bacterial host cells.

Methods for preparing libraries containing diverse population of various types of molecules such as peptides, polypeptides, protein, and fragments thereof are well known in the art and are commercially available (see, for example, Ecker and Crooke. Biotechnology 13:351-360 (1995), and the references cited therein, each of which is incorporated herein by reference).

Where a molecule is a peptide, protein or fragment thereof, the molecule can be produced *in vitro* directly or can be expressed from a nucleic acid, which is produced *in vitro*. Methods of synthetic peptide and nucleic acid chemistry are well known to those skilled in the art.

A library of molecules also can be produced, for example, by constructing a cDNA expression library from mRNA collected from a cell, tissue, organ or organism of interest. Methods for producing such libraries are well known in the art (see, for example, Sambrook et al. Molecular Cloning: A laboratory manual (Cold Spring Harbor Laboratory Press 1989, latest edition)) which is incorporated herein by reference.

Preferably, the peptide encoded by the cDNA is expressed on the surface of a cell or a virus containing the cDNA. For example, cDNA can be cloned into a phage vector wherein, upon expression, the encoded peptide is expressed as a fusion protein on the surface of the phage.

Phage display technology was used to identify peptide and protein sequences that bind Angiostatin protein or Endostatin protein, and also to identify peptides that mimic the effector binding sites of effector molecules that interact with Angiostatin protein and Endostatin protein.

Materials and Methods

Phage Display Libraries

The Ph.D.-7 phage display library (New England BioLabs Cat #8100), a combinatorial library consisting of random peptide 7-mers, was screened for 7 amino acid binding peptides. The Ph.D.-7 phage display library consists of linear 7-mer peptides fused to the pIII coat protein of M13 via a Gly-Gly-Gly-Ser flexible linker. The library contains 2.8×10^9 independent clones. The Ph.D. library is useful for identifying targets requiring binding elements concentrated in a short stretch of amino acids.

The Ph.D.-C7C library (New England BioLabs Cat # 8120) is a combinatorial library consisting of random peptide 7-mers flanked by cysteine residues that under non-denaturing conditions are disulfide bonded resulting in the display of cyclized peptides. In non-reducing conditions, the cysteines form a disulfide bond resulting in each peptide being constrained in a disulfide loop. The library contains 3.7×10^9 independent clones that like the Ph.D.-7 library the peptides are fused to the pIII coat protein of M13 via a Gly-Gly-Gly-Ser flexible linker. Constrained libraries are useful in the identification of structural epitopes. The Ph.D.-C7C library was screened for the binding of 7 amino acid cyclized peptides.

This particular library was selected because it contains representatives of all possible 7 mer sequences and is also a well characterized library. In addition, the short target sequences typically bind with few high affinity interactions that

allow the identification of strong interactions. In summary, the peptides in this library allow for binding such that peptides bind with greater affinity thereby increasing the likelihood of mimicking the natural ligand of the target protein.

Phage Selection

The phage display libraries were screened following the manufacturers' recommendations. Approximately 100µg/ml of recombinant human Angiostatin protein, K1-4 protein, or recombinant human Endostatin protein were coated overnight onto 6-mm tissue culture plates at 4°C in a humidified container. In the first round of panning approximately $2x10^{11}$ phage were incubated on the protein coated plate for 60 minutes at room temperature while rocking gently. The plates were washed 6 times using TBS (50mM Tris-HCl (pH 7.5), 150 mM NaCl) containing 0.1% Tween 20. The binding phage were collected and amplified following elution using 100µg/ml of the target protein. Secondary and tertiary pannings were performed as for the primary screen except the TBS washing buffer contained 0.5% Tween 20.

Sequencing of Angiostatin-Related Binding Peptides

Following the tertiary screening, 10-12 individual colonies of phage-infected bacteria were picked at random, the phage DNA was isolated and subjected to automated dideoxy sequencing. The sequence of the displayed peptides were deduced from the DNA sequence.

Results

Peptides presented below in Tables 7-10 were identified as linear or cyclized peptides that bind Angiostatin protein or Endostatin protein.

Table 7
Linear Peptides Binding Angiostatin Protein

Peptide	Amino Acid Sequence	SEQ ID NO:
A1	E R V N D D T G G G S	SEQ ID NO:3
A3	D R S G A I K G G G S	SEQ ID NO:4
A7	L D R A N V F G G G S	SEQ ID NO:5
A9	S P L G G S E G G G S	SEQ ID NO:6
A10	H A I Y P R H G G G S	SEQ ID NO:7

Table 8
Cyclized Peptides Binding Angiostatin Protein

Peptide	Amino Acid Sequence	SEQ ID NO:
A25	C W S Y E W S K C G G G S	SEQ ID NO:8
A31	C W S L E Q S K C G G G S	SEQ ID NO:9
A35	C W S L E W Q K C G G G S	SEQ ID NO:10
A28	C W S L E T T K C G G G S	SEQ ID NO:11
A33	C W S L E H Q K C G G G S	SEQ ID NO:12
A34	C W S L E I L K C G G G S	SEQ ID NO:13
A30	C W T L E S T K C G G G S	SEQ ID NO:14
A32	C G D M S D R P C G G G S	SEQ ID NO:15

Table 9
Linear Peptides Binding Endostatin Protein

Peptide	Amino Acid Sequence	SEQ ID NO:
E15	H K R P R N N G G G S	SEQ ID NO:16
E12	T K H R A G R G G G S	SEQ ID NO:17
E13	W H R S V W K G G G S	SEQ ID NO:18
E14	S P Q P F E E G G G S	SEQ ID NO:19
E16	F T E P T H K G G G S	SEQ ID NO:20
E17	K D Y A L P P G G D S	SEQ ID NO:21
E18	S K I A P I M G G G S	SEQ ID NO:22
E20	W R Q T R K D G G G S	SEQ ID NO:23
E22	G K P M P P M G G G S	SEQ ID NO:24
E37	C T H W W H K R C G G G S	SEQ ID NO:25

Table 10
Cyclized Peptides Binding Endostatin Protein

Peptide	Amino Acid Sequence	SEQ ID NO:
E41	C S L T P H R Q C G G G S	SEQ ID NO:26
E45	C E K E K P M T C G G G S	SEQ ID NO:27
E48	C A P P G L A R C G G G S	SEQ ID NO:28

EXAMPLE 2

Identification of Binding Peptides that Preferentially Bind Angiostatin *protein over Plasminogen*

Both the linear (Ph.D.-7) and constrained (Ph.D.-C7C) libraries as described in Example 1 were screened for Angiostatin protein specific binding clones: phage that preferentially bind to Angiostatin protein as opposed to plasminogen.

Materials and Methods

Four rounds of screening were performed on both libraries as follows:

Round 1.

- $2x10^{11}$ phage bound to Angiostatin protein coated plate
- plated washed 10 times
- eluted phage with 100μg/ml plasminogen
- plated washed 10 times

•eluted phage with 100μg/ml Angiostatin protein K1-4
•amplified the Angiostatin protein eluted phage

Round 2

•$2x10^{11}$ amplified primary screen Angiostatin protein eluted phage bound to Angiostatin protein coated plate
•plated washed 10 times
•eluted phage with 100μg/ml plasminogen plated washed 10 times eluted phage with 100μg/ml Angiostatin protein K1-4
•amplified the Angiostatin protein eluted phage

Round 3

•$2x10^{11}$ amplified secondary screen Angiostatin protein eluted phage bound to Angiostatin protein coated plate
•plated washed 10 times
•eluted phage with 200μg/ml lys-plasminogen
•plated washed 10 times
•eluted phage with 100μg/ml Angiostatin protein K1-4
•amplified the Angiostatin protein eluted phage

Round 4

•$2x10^{11}$ amplified tertiary screen Angiostatin protein eluted phage bound to Angiostatin protein coated plate
•plated washed 10 times
•eluted phage with 200μg/ml lys-plasminogen
•plated washed 10 times
•eluted phage with 100μg/ml Angiostatin protein K1-4

Eluted phage was titrated and plated at approximately 75 pfu per plate. 24 individual plaques were picked from the

linear (clones PAL 49 to PAL-72) and constrained (clones PAC-73 to PAC-96) libraries and the phage amplified.

Untitrated phage were assayed for their ability to bind to Angiostatin protein and plasminogen in an ELISA assay. Four fold dilutions of phage ranging from 1:4 to 1:65 536 were tested. Several phage were identified as have preferential binding to Angiostatin protein as compared to plasminogen. These phage were selected for further analysis.

Table 11
Linear Peptides that Preferentially Bind to Angiostatin protein over Plasminogen

Peptide	Amino Acid Sequence	SEQ ID NO:
PAL-49	K C C Y Y A K G G G S	SEQ ID NO:31
PAL-51	K C C Y P S A G G G S	SEQ ID NO:32
PAL-54	R Q P P H L H G R G S	SEQ ID NO:33
PAL-56	H K Y I S A T G G G S	SEQ ID NO:34
PAL-66	G T L Q V L S G G G S	SEQ ID NO:35
PAL-69	K C C Y S V G G G G S	SEQ ID NO:36
PAL-70	M S Y Q W S H G G G S	SEQ ID NO:37

Table 12
Cyclized Peptides that Preferentially Bind to Angiostatin protein over Plasminogen

Peptide	Amino Acid Sequence	SEQ ID NO:
PAC-77	C W S L E H S K C G G G S	SEQ ID NO:38
PAC-78	C V H S I E R E C G G G S	SEQ ID NO:39
PAC-82	C Y T L P P K L C G G G S	SEQ ID NO:40
PAC-88	C W S Y E W S K C G G G S	SEQ ID NO:41
PAC-91	C W S L E W Q K C G G G S	SEQ ID NO:42

EXAMPLE 3

Comparison of Angiostatin-Related Binding Peptides using Phage Display Technology and ELISA Assays

A modified ELISA assay was used to determine specificity of binding of the phage to the target protein as recommended by New England BioLabs. Plates coated with the target proteins (Angiostatin protein, Endostatin, plasminogen) were incubated with 4-fold serial dilutions of the phage from

approximately $6.25x10^{10}$ to $2x10^{5}$ phage particles in 96-well plates. The plates were washed 6 times with TBS containing 0.5% Tween 20 followed by incubation with 1:5000 diluted HRP-conjugated anti-M13 antibody (Pharmacia # 27-9411-01). The plates were washed 6 times with TBS containing 0.5% Tween 20 followed by incubation with ABTS (2,2'-AZINO-bis(3-ETHYLBENZ-THIAZOLINE-6 SULFONIC ACID)) Peroxidase substrate solution at room temperature for 10-60 minutes. The plates were read at 410nm using a Molecular Devices Spectra MAX 250 microplate reader and SOFTmax® Pro software.

As shown in Figures 4 and 5. the peptides selected using phage display technology, preferentially bound to target proteins Angiostatin protein over plasminogen.

EXAMPLE 4

Identification of Proteins Bearing Sequence Homology to Peptides Identified as Binding Angiostatin *protein or* Endostatin *protein*

A protein database was searched for proteins that share sequence homology with the peptides identified previously as binding Angiostatin protein and Endostatin protein (A1-A11, E12-E22, A25-A36, E37-E48, PAL-49, 51, 54, 56, 66, 69, 70, and PAC-77, 78, 82, 88 and 91). Because of the short length of the peptides (the shorter the sequence used in a search the lower the specificity) a significant number of proteins that shared homology with the identified peptides were found. A selection of the peptides with the highest homology or which appear to be biologically interesting are provided in Tables 13 and 14. The selection of proteins with the highest homology with the PAL and PAC peptides or which appear to be biologically interesting are provided in Tables 15 and 16.

The homology searches were conducted using the FASTA sequence similarity search. The peptide sequences were queried against the SwissProt data base using the default setting (ktup 2, and BLOSUM50 for the scoring matrix). The searches were conduced using the world wide web at site http://www.fasta.genome.ad.jp/. as described by W.R. Pearson &

D.J. Lipman PNAS 85:2444-2448 (1988), which is incorporated in its entirety herein by reference.

Table 13

Search Results for Homologies Against the 7 amino acid Peptide Sequences

Protein Containing Homologous Sequence	Source	Level of Homology
Human possible global transcription activator	Mammalian (Human)	7 of 7 amino acids with A9
phosphopentomutase	Bacteria	6 of 7 amino acids plus 1 conserved with A3
ribonuclease RH precursor	Fungi	6 of 7 amino acids plus 1 conserved with A35
soybean early nodulin	Soybean	6 of 7 amino acids plus 1 conserved with A25
JNK activating kinase 1	Mammalian (Human)	6 of 7 amino acids with A3
IL-12 beta chain	Mammalian (Mouse)	6 of 7 amino acids with A34
glutathione reductase	Spinach (Chloroplast)	5 of 7 amino acids plus 2 conserved with A3
Soy bean trypsin inhibitor (kunitz)	Soybean	5 of 7 plus 1 conserved amino acid with A1
fibroblast growth factor-6	Human Mouse	5 of 7 plus 2 conserved amino acid with E18
chemotaxis protein	Bacteria	5 of 7 plus 1 conserved amino acid with E12
Annexin XI	Human Rabbit Mouse	5 of 7 plus 1 conserved amino acid with E22
WEE 1	Yeast	5 of 7 plus 1 conserved amino acid with A32
RAS suppressor protein 1	Mammalian (Human)	5 of 7 A32
ATP synthase gamma chain	Bacteria	5 of 7 A32
Thioredoxin	Bacteria	5 of 7 with A1

Table 14
Search Results for Homologies Against the 7 Amino Acid Peptide Plus the GGGS Linker Sequence

Protein Containing Homologous Sequence	Source	Level of Homology
Collagenese*	Bacteria	9 of 13 with A32
Glycoprotein B-1 precursor	Virus	8 of 11 with E12
Dehydroquinate dehydratase	Bacteria	8 of 11 with E16
Complement component C8 beta chain*	Mammalian (Rat)	8 of 13 with plus 2 conserved with A25
Ornithine decarboxylase antizyme	Mammalian (Hamster)	7 of 11 plus 2 conserved with E13
adenylate cyclase	Bacteria	7 of 11 plus 2 conserved with E20
ATP synthase alpha chain	Bacteria	6 of 11 plus 4 conserved with A1
ATP synthase alpha chain	Bacteria	6 of 11 plus 1 conserved with A10

* The constrained peptides were 13 amino acids because of the 2 cysteines i.e. C-XXXXXXX-CGGGS.

An additional selection of peptides having high homology and appearing biologically interesting, are provided in Tables 15 and 16.

Table 15
Search Results for Homologies Against the
7 amino acid Peptide Sequences

Protein Containing Homologous Sequence	Source	Level of Homology
FLIY PROTEIN PRECURSOR	E. coli bacteria	6 out of 7 plus 1 conserved with PAL-66
HEMAGGLUTININ-NEURAMINIDASE	Para influenza (Flu Virus)	6 out of 7 plus 1 conserved with PAL-66
HISTONE H1	Drosphila (fruit fly)	6 out of 7 PAL-56
ADP, ATP CARRIER PROTEIN 2 PRECURCOR	Wheat (plant)	6 out of 7 plus with PAL-66
RIBONUCLEASE 1 PRECURSOR	Thale-cress (plant)	5 out of 7 plus 2 conserved with PAL-51
CALRETININ	Mouse (mammalian)	5 out of 7 plus 2 conserved with PAL-53
HYPOTHETICAL 64.1 KD ZINC FINGER PROTEIN	Yeast (eukaryotic)	5 out of 7 plus 2 conserved with PAL-70
T-CELL SURFACE ANTIGEN CD2 PRECURSOR	Rat and mouse (mammalian)	5 out of 7 plus 2 conserved with PAL-70
ATP SYNTHASE C CHAIN	Bacillus alcalophilus (bacteria)	5 out of 7 plus 1 conserved with PAL-66
CELL DIVISION INHIBITOR MINC.	E. coli (bacteria)	5 out of 7 plus 1 conserved with PAL-56
CELL DIVISION CONTROL PROTEIN 45	Yeast (eukaryotic)	5 out of 7 plus 1 conserved with PAC-78
FETAL BRAIN PROTEIN	Human (mammalian)	5 out of 7 plus 1 conserved with PAL-53
HOLLIDAY JUNCTION DNA HELICASE RUVA	Pseudomonas aeruginosa (bacteria)	5 out of 7 plus 1 conserved with PAL-53

Table 16
Search Results for Homologies Against the 7 Amino Acids Plus the GGGS Linker Sequence

Protein Containing Homologous Sequence	Source	Level of Homology
CELL WALL PROTEIN QID3 PRECURSOR	Trichoderma harzianum (eukaryotic)	8 of 13 plus 2 conserved with PAC-82*
HYDROPHOBIN PRESURSOR (RODLET PROTEIN)	Aspergillus fumigatus (Eukaryotic)	8 of 11 plus 1 conserved with PAL-66
CARBOXYPEPTIDASE Y PRECURSOR	Yeast (eukaryotic)	7 of 13 plus 4 conserved with PAC-78*
NODULIN 24 PRECURSOR	Soy Bean (plant)	7 of 11 plus 2 conserved with PAL-51
HYPOTHETICAL ABC TRANSPORTER PERMEASE PRO	E. coli (bacteria)	7 of 11 plus 1 conserved with PAL-66

* The constrained peptides were 13 amino acids because of the 2 cysteines i.e. C-XXXXXXX-CGGGS.

EXAMPLE 5

Angiostatin Protein and Endostatin Protein Interaction with Tropomyosin

Based on the rationale that the peptides that bound to Angiostatin protein or ENDOSTATIN protein represent the binding domains of larger proteins that bind Angiostatin and/or Endostatin proteins, it was theorized that Angiostatin protein and/or Endostatin protein binding peptides and proteins would share common motifs that are recognized by corresponding anti-peptide antibodies. Consequently, a HUVEC expression library was screened with antibodies against the A10 and E37 peptides (A10 peptide binds Angiostatin protein while E37 peptide binds Endostatin protein, see Examples 1 and 2). In doing so, multiple clones that code for human tropomyosin were isolated.

Though not wishing to be bound by the following theory, it is believed that proteins and peptides that bind Endostatin protein comprise tropomyosin and, that through this

interaction, exert their angiogenic effects. Tropomyosin was identified based on the DNA sequence of the isolated clone. The 5 prime and 3 prime ends of the clone being recognized by the A10 and E37 antibodies were determined by routine DNA sequencing techniques. The obtained sequences were then 'BLASTED' against the GeneBank database (of DNA sequences) and a high degree of sequence similarity was observed between both the 5 prime and 3 prime end sequences of our clone and human tropomyosin mRNA. The BLAST searches were conducted using the WWW site at *http://www.blast.genome.ad.jpl* using the default setting (scoring matrix BLOSUM62). Results of the BLAST searches are provided below.

5 Prime End Blast Results

High Probability
Sequences producing High-scoring Segment Pairs: Score P(N) N
gb:HUMTRO Human tropomyosin mRNA, complete cds. 342 2.1e-20 1
gb:S78854 alpha-tropomyosin [rabbits, New Zealand whi... 309 1.1e-17 1
gb:SSCATROP S.scrofa mRNA for cardiac alpha tropomyosin. 302 4.3e-17 1
gb:HUMTROPA2 Human skeletal muscle alpha-tropomyosin (hT... 297 1.1e-16 1
gb:RATTMBR1A Rat brain alpha-tropomyosin (TMBr-1) mRNA, ... 293 2.4e-16 1
gb:MMPTMA M.musculus mRNA for skeletal muscle alpha t... 293 2.4e-16 1
gb:MUSTRO2IS Mouse tropomyosin isoform 2 mRNA, complete ... 293 2.5e-16 1
gb:RATTRO2A Rat alpha-tropomyosin 2 mRNA, complete cds. 293 2.5e-16 1
gb:RATTRO3A Rat alpha-tropomyosin 3 mRNA, complete cds. 293 2.5e-16 1
gb:RATTMA1 Rat alpha-tropomyosin gene, exons 1-3. 279 3.9e-15 1
gb:AR008277 Sequence 3 from patent US 5753446. 242 8.7e-15 2
gb:AR008281 Sequence 11 from patent US 5753446. 242 8.7e-15 2
gb:RATTROPA Rat smooth muscle alpha-tropomyosin mRNA, c... 268 1.3e-13 1
gb:RNTROASM Rat mRNA for smooth muscle alpha-tropomyosin. 268 1.3e-13 1
gb:A62300 Sequence 3 from Patent WO9712982. 228 1.2e-12 2
gb:HUMTRPMYO Human tropomyosin-1 (TM-beta) mRNA, complet..246 3.1e-11 1
gb:HUMTM1E H.sapiens epithelial tropomyosin (TM1) mRNA... 246 3.1e-11 1
gb:RATTRO01 Rat skeletal muscle beta-tropomyosin and fi... 237 2.2e-10 1
gb:A62298 Sequence 1 from Patent WO9712982. 234 4.7e-10 1
gb:AR018138 Sequence 1 from patent US 5780609. 234 4.8e-10 1

gb:AR008278 Sequence 5 from patent US 5753446. 230 1.1e-09 1
gb:AMTTROPOX Ambystoma mexicanum tropomyosin mRNA, compl. 229 1.3e-09 1
gb:AB005878 Nicotiana tabacum mRNA for BYJ15, partial cds. 228 1.4e-09 1
gb:AB005879 Nicotiana tabacum mRNA for BYJ6, partial cds. 228 1.4e-09 1
gb:DMELAST D.melanogaster mRNA for elastin-like protein. 228 1.4e-09 1
gb:TEZ86120 T.evansi mRNA, clone Q16R1. 228 1.6e-09 1
gb:MMBTROP M.musculus mRNA for beta-tropomyosin. 228 1.6e-09 1
gb:MUSBETATRO Mouse beta-tropomyosin 2 mRNA, complete cds. 228 1.6e-09 1
gb:MMTPMYOB Mus musculus gene for beta-tropomyosin. 228 1.6e-09 1
gb:AB002449 Homo sapiens mRNA from chromosome 5q21-22. ... 228 1.6e-09 1
gb:OSCHINDPR O.sativa mRNA for chilling-inducible protein. 228 1.7e-09 1
gb:I14842 Sequence 10 from patent US 5455167. 228 1.7e-09 1
gb:I65496 Sequence 6 from patent US 5667997. 228 1.7e-09 1
gb:ATCYC2B A.thaliana (Columbia) cyc2b mRAN for cyclin... 228 1.7e-09 1
gb:I79511 Sequence 2 from patent US 5707809. 228 1.7e-09 1
gb:AF060519 Cuphea hookeriana 3-ketoacyl-ACP synthase (... 228 1.7e-09 1
gb:MUSTROB Mouse skeletal muscle beta tropomyosin mRNA... 228 1.7e-09 1
gb:AF058696 Homo sapiens cell cycle regulatory protein ... 228 1.8e-09 1
gb:MSASET2MR M.sativa mRNA for ASET2. 220 8.9e-09 1
gb:AB005877 Nicotiana tabacum mRNA for BYJ14, partial cds. 219 9.0e-09 1
gb:S71728 truncated protein {frame 1, multiple clonin... 198 9.4e-09 1
gb:I50126 Sequence 19 from patent US 5641876. 198 1.1e-08 1
gb:I50132 Sequence 25 from patent US 5641876. 198 1.1e-08 1
gb:I50128 Sequence 21 from patent US 5641876. 198 1.1e-08 1
gb:I50133 Sequence 26 from patent US 5641876. 198 1.2e-08 1
gb:PSP54MRNA Pisum sativum mRNA for P54 protein. 218 1.4e-08 1
gb:CHKTRPMYB Chicken skeletal muscle beta-tropomyosin mR... 216 2.1e-08 1
gb:CHKTROBRT1 Chicken beta-tropomyosin 1 (BRT-1) mRNA, co... 216 2.2e-08 1
gb:I82448 Sequence 60 from patent US 5712143. 213 2.2e-08 1
gb:CHKTROSS01 Chicken tropomyosin beta subunit gene, exon... 216 2.2e-08 1
gb:AR013938 Sequence 1 from patent US 5773225. 193 3.4e-08 1
gb:AR016808 Sequence 33 from patent US 5777200. 213 3.7e-08 1
gb:AR020834 Sequence 33 from patent US 5789214. 213 3.7e-08 1
gb:I38463 Sequence 33 from patent US 5614395. 213 3.7e-08 1
gb:I56938 Sequence 33 from patent US 5650505. 213 3.7e-08 1
gb:I59804 Sequence 33 from patent US 5654414. 213 3.7e-08 1
gb:I75131 Sequence 33 from patent US 5689044. 213 3.7e-08 1
gb:NTZ82982 N.tabacum mRNA for caffeoyl-CoA O-methyltra... 213 3.9e-08 1
gb:MZEAGAMOU Zea maize AGAMOUS homologue mRNA, complete 213 3.9e-08 1

gb:AF030383 Cucumis melo var. markuwa Markino ADP-gluco... 213 4.0e-08 1
gb:CLCYCBMR C.longicaudatus mRNA for cyclin B. 213 4.0e-08 1
gb:CPU69698 Cryptosporidium parvum heat shock protein 7... 211 6.2e-08 1
gb:S81027 Msr-110=EN protein binding gene/engrailed n... 208 1.1e-07 1
gb:HUMTROPA Human fibroblast muscle-type tropomyosin mR... 207 1.3e-07 1
gb:CHKATRO Chicken fast-twitch alpha-tropomyosin mRNA.... 207 1.3e-07 1
gb:HSTPMYOB Human mRNA for skeletal beta-tropomyosin. 207 1.3e-07 1
gb:QULTROAB Japanese quail alpha-tropomyosin mRNA, comp... 207 1.3e-07 1
gb:I94990 Sequence 9 from patent US 5731411. 188 1.3e-07 1
gb:CHKAFTROP1 Chicken alpha-tropomyosin gene, exons 1a, 2... 207 1.4e-07 1
gb:GGTME1A2A G.gallus gene for tropomyosin (alpha isofor... 207 1.4e-07 1
gb:CCTPMY01 Quail gene for alpha-tropomyosin, exons 1-3... 207 1.4e-07 1
gb:QULTROAA Japanese quail alpha-tropomyosin mRNA, comp... 206 1.7e-07 1
gb:MAU29167 Mesocricetus auratus tropomyosin-1 mRNA, co... 205 2.0e-07 1
gb:S71730 influenza virus hemagglutinin 5' epitope ta... 198 2.0e-07 1
gb:DMIPOU D.melanogaster I-POU mRNA for a POU-domain ... 205 2.1e-07 1
gb:SYNPLKRA Cloning vector pUC128 DNA, polylinker region. 198 2.4e-07 1
gb:SYNPLKRB Cloning vector pUC129 DNA, polylinker region. 198 2.6e-07 1
gb:S71742 influenza virus hemagglutinin 5' epitope ta... 198 2.8e-07 1
gb:A59058 Sequence 1 from Patent WO9703200. 203 3.1e-07 1
gb:GGINTB3 G.gallus mRNA for integrin beta3. 203 3.2e-07 1
gb:STRSTRH Streptococcus pneumoniae beta-N-acetylhexos... 203 3.3e-07 1
gb:S71745 influenza virus hemagglutinin 5' epitope ta... 198 3.5e-07 1
gb:RANATROA R.temporaria skeletal muscle alpha-tropomyo... 201 4.5e-07 1
gb:AF067142 Cloning vector pSFI polylinker, complete po... 198 4.6e-07 1
gb:AR016514 Sequence 24 from patent US 5776746. 198 6.0e-07 1
gb:CVU61229 Cloning vector pKRX, complete sequence. 199 7.3e-07 1
gb:AR008443 Sequence 70 from patent US 5753488. 198 7.8e-07 1
gb:I87435 Sequence 70 from patent US 5703221. 198 7.8e-07 1
gb:PFL17187 Platichthys flesus Ki-ras gene (exons 1, 2,... 198 7.9e-07 1
gb:DMFRIZZ3 D. melanogaster frizzled gene exons 3 and 4. 198 8.4e-07 1
gb:SMOFMTRMYA Salmo salar fast myotomal muscle tropomyosi... 198 8.4e-07 1
gb:CHKTROPB Chicken tropomyosin (clone CTm7) mRNA, comp... 198 8.5e-07 1
gb:A45456 Sequence 1 from Patent EP0607925. 198 8.5e-07 1
gb:I17500 Sequence 1 from patent US 5489743. 198 8.5e-07 1
gb:D88747 Arabidopsis thaliana mRNA for AR401, comple... 198 8.6e-07 1
gb:NTY14032 Nicotiana tabacum mRNA for ferredoxin-NADP ... 198 8.6e-07 1
gb:CHKTROPA Chicken tropomyosin (clone CTm4) mRNA, comp... 198 8.6e-07 1
gb:MPCAM M.pyrifera mRNA for calmodulin. 198 8.7e-07 1

gb:PFL17188 Platichthys flesus Ha-ras gene (exons 1 to 4). 198 8.7e-07 1
gb:ORZPC Oryzias latipes mRNA for photolyase. comple... 198 8.8e-07 1

3 Prime End Blast Results

Sequences producing High-scoring Segment Pairs: Score P(N) N
gb:HSAJ147 Homo sapiens mRNA for alpha-tropomyosin (3'... 700 1.9e-110 4
gb:HSALPTROP Homo sapiens mRNA for alpha-tropomyosin. 1055 1.4e-96 2
gb:HSTROISOA H.sapiens tropomyosin isoform mRNA, complet... 1012 2.9e-76 1
gb:MUSTRO2IS Mouse tropomyosin isoform 2 mRNA, complete ... 226 4.3e-33 4
gb:RATTMA6 Rat alpha-tropomyosin gene, exon 12. 267 1.9e-28 3
gb:RATTRO5A Rat alpha-tropomyosin 5a mRNA, complete cds. 267 7.9e-24 3
gb:RATTRO5B Rat alpha-tropomyosin 5b mRNA, complete cds. 267 7.9e-24 3
gb:RATTRO2A Rat alpha-tropomyosin 2 mRNA, complete cds. 267 8.7e-24 3
gb:RATTRO3A Rat alpha-tropomyosin 3 mRNA, complete cds. 267 8.7e-24 3
gb:RATTMBR3A Rat brain alpha-tropomyosin (TMBr-3) mRNA, ... 267 2.2e-23 3
gb:RATTROPA Rat smooth muscle alpha-tropomyosin mRNA, c... 206 5.9e-18 3
gb:RNTROASM Rat mRNA for smooth muscle alpha-tropomyosin. 206 5.9e-18 3
gb:CHKAFTROP6 Chicken alpha-tropomyosin gene, exon 9c, 9d... 273 2.9e-12 1
gb:GGTME9C9D G.gallus gene for tropomyosin (alpha isofor... 273 2.9e-12 1
gb:HSTROPM Human mRNA (exon 6-9 part.) for smooth musc... 224 3.6e-08 1
gb:HUMTROPA4 Human skeletal muscle alpha-tropomyosin (hT... 224 3.6e-08 1
gb:HUMTRO Human tropomyosin mRNA, complete cds. 224 3.7e-08 1
gb:CCTPMY13 Quail gene for alpha-tropomyosin, exon 13. 146 0.13 1
gb:DMU66884 Drosophila melanogaster cubitus interruptus... 133 0.83 1
gb:CEZK666 Caenorhabditis elegans cosmid ZK666, comple... 132 0.88 1
gb:AC004002 Human BAC clone RG356F09 from 7p21, complet... 132 0.88 1

EXAMPLE 6

Identification of Peptides Capable of Binding Endostatin protein

As a result of the findings of the above experiments, nine clones from a HUVEC lambda Uni-ZAP XR cDNA library were purified based on the observation that they express a protein that is recognized by polyclonal antibodies raised against the A10 and E37 peptide (see Examples 1 and 2). Sequencing data showed that 6 of the 9 code for tropomyosin. The sequencing reaction for one clone failed but restriction analysis showed that it also

codes for tropomyosin. Thus 7 of the 9 clones code for tropomyosin.

One clone codes for human glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The GenBank accession number for this sequence is HUMG3 PDC.

The other clone shares homology with (cr36c10.x1) Jia bone marrow stroma Homo sapiens cDNA clone. The GenBank accession number for this sequence is AI755161.

Analysis of the DNA sequences has shown that the 5' end of one clone, PL26/2, codes for GAPDH while the 3' end codes for an SH 3 domain binding glutamic acid-rich-like protein (SH# BGRL. GeneBank accession number AFO42081).

Though not wishing to be bound by the following theory, it is thought that it is the anti-E37 antibodies recognize tropomyosin with greater affinity than the anti-A10.

Using an antibody that recognizes tropomyosin the interaction of tropomyosin with Endostatin protein is analyzed. Also, FACS analysis is conducted to demonstrate the location of tropomyosin and to show its hypothesized location on the surface of endothelial cells. Additional experiments blocking the binding of Endostatin protein to the surface of HUVEC cells and comparable assays for Angiostatin protein are performed. Further experiments involving immunoprecipitations using antibodies against Angiostatin protein and/or Endostatin protein are performed to confirm a receptor/protein pairing based on resulting tropomyosin co-precipitating and vise versa.

Confirmation of binding, for example binding of Endostatin protein to tropomyosin is done by expressing Endostatin protein as a glutathione S-transferase (GST) fusion protein in E. coli. The Endostatin -GST is bound to an affinity matrix and incubated with HUVEC extracts. Following washing, the proteins are eluted and run on an SDS-PAGE gel and western blotted probing with anti-tropomyosin antibodies. Interaction of Endostatin protein and tropomyosin results in the detection of tropomyosin on the western blot. The reverse of this experiment is also conducted using tropomyosin-GST and a crude Endostatin protein containing lysate (Picia broth perhaps).

In vivo experiments for determining the role of tropomyosin involved in the activity of Endostatin protein with regard to angiogenesis are also performed. In one such experiment, anti-tropomyosin antibodies are co-administered with Endostatin protein. Confirmation of tropomyosin's interaction with Endostatin protein results from a negation of anti-tumor activity typically resulting from Angiostatin protein or Endostatin protein. In another type of *in vivo* experiment, tropomyosin is recombinantly expressed and co-administered with either Angiostatin protein or Endostatin protein to observe the effect on anti-tumor activity usually associated with Angiostatin protein or Endostatin protein

In another experiment, labeled Angiostatin protein or Endostatin protein (i.e. biotin-labeled Endostatin protein) is administered to tumor bearing mice in order to determine the fate of the labeled protein (i.e. binding sites). In another aspect of the present invention, immunohistochemicial techniques are employed to see if the labeled protein and tropomyosin co-localize. As is known in the art, tropomyosin is involved in the formation of fibers that play a key role in cell shape and motility and therefore in yet another aspect of the invention, immunoflorescence is used to study the effects of Angiostatin protein and/or Endostatin protein on cellular organization normally controlled by tropomyosin.

EXAMPLE 7

Interaction of Laminin Protein and Angiostatin protein

The following experiment was conducted in order to identify a potential receptor, or molecule comprising a receptor or binding partner for Angiostatin protein.

Materials and Methods

Peptides obtained by biopanning against Angiostatin protein were edited to remove multiple copies of sequences. This left the following sequences, arranged into specific classes of sequences.

I. Data

Table 17
Linear Peptide Sequences Capable
of Binding Angiostatin protein

Peptide	Amino Acid Sequence	SEQ ID NO:
PAL-49-M	KCCYYAK	SEQ ID NO:82
PAL-50-M	KCCYSVG	SEQ ID NO:83
PAL-51-M	KCCYPSA	SEQ ID NO:84
PAL-56-M	HKYISAT	SEQ ID NO:85
PAL-53-M	RQPPHLH	SEQ ID NO:86
PAL-66-M	GTLQVLS	SEQ ID NO:87
PAL-70-M	MSYQWSH	SEQ ID NO:88
A1-M	ERVNDDT	SEQ ID NO:89
A3-M	DRSGAIK	SEQ ID NO:90
A7-M	LDRANVF	SEQ ID NO:91
A9-M	SPLGGSE	SEQ ID NO:92
A10-M	HAIYPRH	SEQ ID NO:93

Table 18
Cyclized Peptide Sequences Capable
of Binding Angiostatin protein

Peptide	Amino Acid Sequence	SEQ ID NO:
PAC-77-M	C-WSLEHSK-C	SEQ ID NO:94
A30-M	WTLESTK	SEQ ID NO:95
PAC-82-M	YTLPP-KL	SEQ ID NO:96
PAC-88-M	WSYEWSK	SEQ ID NO:97
PAC-91-M	WSLEWQK	SEQ ID NO:98
A31-M	WSLEQSK	SEQ ID NO:99
A28-M	WSLETTK	SEQ ID NO:100
A33-M	WSLEHQK	SEQ ID NO:101
PAC-78-M	VHSIERE	SEQ ID NO:102
A32-M	GDMSDRP	SEQ ID NO:103

II. Data Base Searches

The obvious searches of sequence data bases did not identify any human proteins that were potential targets of Angiostatin protein. In particular, searches were made for proteins containing the following sequences:

WSLE	SEQ ID NO:104
WTLE	SEQ ID NO:105
WSYE	SEQ ID NO:106
KCCY	SEQ ID NO:107
TLQVL	SEQ ID NO:108

None of these motifs were present in fibrinogen, the natural substrate for plasminogen. A closer look for fibrinogen peptides with similarity to Angiostatin protein-selected peptides was carried out. As outlined below, this search identified a number of places in the fibrinogen alpha chain with significant similarity to the selected peptides.

Because of the presence of fibrinogen sequences with similarities to the selected peptides, additional data base searches were made and one, ERVN (SEQ ID NO: 109) identified a tetrapeptide present in the laminin beta-1 chain. However, a closer look at laminin provides additional weight to the possibility that Angiostatin protein binds laminin beta-1 protein (see below).

III. Fibrinogen

The natural substrate for plasminogen is fibrin. Several studies have shown that fragments of plasminogen containing intact kringle domains bind to fibrin. For instance, Wu et al. (J. Biol. Chem. 265; 19658-64; 1990) showed that a plasminogen fragment containing kringles 1-3 binds to fibrin but with affinity less than a fragment containing kringles 1-5. They showed that kringle 4 does not bind to fibrin. The binding of plasminogen to fibrin involves interactions with lysines on fibrin and can be inhibited by lysine analogues. Only kringle 3 appears to be devoid of a lysine-binding motif (Cao et al., J. Biol. Chem.271; 29. 461-29467; 1996)

The large number of lysines in the Angiostatin protein selected peptides is consistent with the known lysine-binding properties of plasminogen and plasminogen fragments containing intact kringle domains (lysine affinity is k4>k1>k2>k3 (none)). The plasminogen-binding sites on fibrin have not yet been identified (for example see Weisel et al., 1994; J. Mol. Biol. 235; 1117-1135). However, there are a number of sequences in fibrinogen with significant homology to the selected peptides.

These sequences are potentially involved in plasminogen binding and include:

Fibrinogen alpha 91

SLFEYGK	SEQ ID NO: 110
WSLEWQK	SEQ ID NO: 98

Fibrinogen alpha 433

LVTSK	SEQ ID NO:111
WSLETTK	SEQ ID NO:100
WSYEWSK	SEQ ID NO:97
WSLEQSK	SEQ ID NO:99
WTLESTK	SEQ ID NO:95

Fibrinogen alpha 477

EVTK	SEQ ID NO:112
WSLETTK	SEQ ID NO:100
WSYEWSK	SEQ ID NO:97
WSLEQSK	SEQ ID NO:99
WTLESTK	SEQ ID NO:95

These results provide significant and important information about the plasminogen binding sites on fibrinogen.

IV. Laminin beta-1 Chain

Laminin beta-1 chain is 1786 amino acids long, making the odds of it having any tetrapeptide by random chance about 1%. The amino acid sequence of laminin protein is set forth in Figure 6A (SEQ ID NO: 113) and the nucleotide sequence is set forth in Figure 6B (SEQ ID NO: 114). There are three motifs corresponding to Angiostatin protein selected peptides in a single 60 amino acid long region. (See Figure 7).

The convergence of three putative contact motifs in a very small region of laminin strongly supports the possibility that Angiostatin protein binds to laminin protein.

V. Laminin beta-1 Chain Domain Structure

Laminin is a heterotremeric molecule with one each of the alpha, beta and gamma chains making up the molecule. The three chains form triple-helical coiled-coils at several places within the molecule. The putatative binding sites are in a portion of the laminin beta-1 chain designated as Domain II. The molecule is a complex, multi-domain component of the basement membrane organized as follows:

Amino Acid Range	Characterization
1-21	signal sequence
22-270	N-terminal domain
271-540	4=1/2 EGF-like domains (4 complete, 1 partial)
541-771	laminin domain IV
772-1178	8 EGF like domains
1179-1397	domain II
	1216-1315 coiled-coil (approx 150 Angstroms long)
	1353-1388 coiled-coil (approx 54 Angstroms long)
1398-1430	domain alpha
1431-1786	domain I
	1442-1481 coiled-coil (approx 510 Angstroms long)

The putative binding sites are in domain II. The first two sites are in the part of domain II between the two coiled coils (the second binding site is just prior to the start of the second coiled coil). The third site is at the very end, but within, the second coiled coil region (that is consistent with the breaks in the this consensus sequence – to be expected in an alpha helical region). Consequently, sites 2 and 3 are as much as 55 Angstroms apart, separated by most of the coiled coil.

VI. Mouse Laminin

Mouse laminin is commercially available. Mouse laminin beta-1 chain is 90% identical and 94% similar to human laminin. In the putative binding sites, only the first amino acid of the first site is different. Consequently, if Angiostatin protein binds to human laminin, it will most likely also bind to mouse laminin.

VII. Molecular Mechanism of Angiostatin protein

Screening of a phage-displayed library of peptides has identified peptides with similarity to sequences in fibrinogen

and laminin beta-1 chain protein. It is believed that Angiostatin protein has an affinity for laminin beta 1 chain protein. It is further believed that laminin protein comprises a receptor, or a molecule comprising a receptor or binding partner of Angiostatin protein.

Using an antibody that recognizes laminin protein the interaction of laminin protein with Angiostatin protein is analyzed. Also, FACS analysis is conducted to demonstrate the location of laminin protein and to show its location on the surface of endothelial cells. Additional experiments blocking the binding of Angiostatin protein to the surface of HUVEC cells and comparable assays for Endostatin protein are performed. Further experiments involving immunoprecipitations using antibodies against Angiostatin protein and/or Endostatin protein are performed to confirm a receptor/protein pairing based on resulting laminin protein co-precipitating and vice versa.

EXAMPLE 8

In Vitro Interaction of Tropomyosin and Endostatin Protein

The following experiment was conducted in order to assess the interaction of tropomyosin and Endostatin protein.

Materials and Methods

In this experiment the wells of a 96 well plate were either coated with 5 μg/ml of Endostatin protein or BSA protein

The wells were incubated in the presence of serial dilutions of *E coli* lysate that either containing or not containing tropomyosin protein.

The wells were washed and incubated with anti-tropomyosin antibodies followed by anti-mouse IgG AP conjugated antibodies and developed using KPL Blue Phos microwell phosphatase solution. The OD 635 was measured after a 1 hour incubation at RT and the results plotted on a graph (see Figure 8).

Four conditions were tested:

1) BSA coated well + *E. coli* lysate containing tropomyosin.

2) Endostatin protein coated well + E. coli lysate containing tropomyosin

3) Endostatin protein coated well + E. coli lysate containing no tropomyosin

4) BSA coated well + E. coli lysate containing no tropomyosin

Results and Conclusion

As shown by the results in the graph, (Figure 8) when *E. coli* lysate containing tropomyosin is incubated on a Endostatin protein coated well (sample 2) considerably more tropomyosin binds than when the well is BSA coated or when there is no tropomyosin contained in the *E.coli* lysate. The higher the OD635 the more tropomyosin is binding the plate.

The results of this experiment therefore demonstrate positive binding interaction between tropomyosin and Endostatin protein.

EXAMPLE 9

In Vivo Interaction of the E37 Peptide and Endostatin Protein

The following experiment was conducted to determine the interaction of E37 peptide and Endostatin protein.

Materials and Methods

A brief description of the B16BL6 experimental metastasis assay is provided below. This assay is well known to those skilled in the art, for more detail see United States Patent Application Serial No. 09/413,049 and International Patent Application number PCT/US99/11418.

$5x10^4$ B16BL6 cells in 200µl of PBS were injected into C57BL/6J mice via the tail vein. Three days post injection treatment was started. There were 8 groups receiving treatments as described below:

1) citrate phosphate/PBS buffer

2) 30μg Endostatin protein (in 100μl PBS)

3) 30μg Endostatin protein (in 200μl PBS)

4) 30μg Endostatin protein + 50x molar excess of control peptide (in 200μl PBS)

5) 30μg Endostatin protein + 250x molar excess of control peptide (in 200μl PBS)

6) 30μg Endostatin protein + 10x molar excess of E37 peptide (in 200μl PBS)

7) 30μg Endostatin protein + 50x molar excess of E37 peptide (in 200μl PBS)

8) 30μg Endostatin protein + 250x molar excess of E37 peptide (in 200μl PBS)

Doses were administered daily for eleven days posterior to the thoracic cavity subcutaneously. All mice were sacrificed, the lungs removed and the number of surface metastases in each animal were counted under a stereo microscope.

Results

A summary of the results of this experiment are listed below, and detailed results of this experiment are provided in Figure 9 wherein the number of metastases on each lung is listed, as well as the mean, standard deviation, T/C and the results of a 2-tailed T-test. The results are graphically described in Figure 10.

Endostatin protein alone inhibited the formation of lung metastasis by ~79%

Endostatin protein + 50 fold molar excess of the negative control peptide inhibited the formation of lung metastasis by ~79%

Endostatin protein + 250 fold molar excess of the negative control peptide inhibited the formation of lung metastasis by ~73%

Endostatin protein + 10 fold molar excess of the E37 peptide inhibited the formation of lung metastasis by ~76%

Endostatin protein + 50 fold molar excess of the E37 peptide inhibited the formation of lung metastasis by ~61%

Endostatin protein + 250 fold molar excess of the E37 peptide inhibited the formation of lung metastasis by ~21%

Conclusion

The current experiment indicates that the E37 peptide and Endostatin protein interact *in vivo* . We make this assumption on the basis that in the presence of the E37 peptide (but not a random control peptide) the anti-tumor activity of Endostatin protein is inhibited. We conclude from this that the reduced activity of the Endostatin protein in the presence of the E37 peptide is the result of the E37 peptide binding to the Endostatin protein. The effect of the E37 peptide binding to Endostatin protein is that Endostatin can no longer bind its receptor/binding protein, which we contend is tropomyosin.

It should be understood that the foregoing relates only to preferred embodiments of the present invention, and that numerous modifications or alterations may be made therein without departing from the spirit and the scope of the invention as set forth in the appended claims. The references cited throughout are hereby incorporated by reference in their entireties.

We claim:

1. An angiogenesis-inhibiting protein receptor comprising an amino acid sequence comprising SEQ ID NOS: 3-15, SEQ ID NOS: 31-42 and SEQ ID NOS: 82-113, wherein the angiogenesis-inhibiting protein comprises a protein having a molecular weight of between approximately 38 kilodaltons and 45 kilodaltons as determined by reducing polyacrylamide gel electrophoresis, and having an amino acid sequence substantially similar to that of a plasminogen fragment beginning at approximately amino acid 98 of a plasminogen molecule.

2. A nucleic acid coding the receptor of Claim 1, having a nucleotide sequence comprising SEQ. ID NOS: 43-55 and SEQ ID NOS: 69-80.

3. The angiogenesis-inhibiting protein receptor of Claim 1, wherein the receptor comprises laminin protein, tropomyosin, and active fragments and homologs thereof.

4. A method of increasing angiogenesis in an individual, comprising administering to an individual an angiogenesis increasing amount of a receptor of Claim 1.

5. A method of identifying an angiogenesis-inhibiting protein receptor comprising:

(a) obtaining a peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOS: 3-15, SEQ ID NOS: 31-42 and SEQ ID NOS: 82-113;

(b) testing said peptide for an ability to bind angiogenesis-inhibiting protein,

(c) testing said peptide for an ability to affect angiogenesis-inhibiting protein-mediated angiogenesis, wherein the ability to bind an angiogenesis-inhibiting protein and affect angiogenesis-inhibiting protein-mediated angiogenesis identifies the angiogenesis-inhibiting protein receptor,

(d) wherein the angiogenesis-inhibiting protein comprises a protein having a molecular weight of between approximately 38 kilodaltons and 45 kilodaltons as determined by reducing polyacrylamide gel electrophoresis, and having an amino acid sequence substantially similar to that of a plasminogen fragment beginning at approximately amino acid 98 of a plasminogen molecule.

6. The method of Claim 5, wherein the angiogenesis-inhibiting protein receptor comprises laminin protein, tropomyosin, and active fragments and homologs thereof.

7. An angiogenesis-inhibiting protein receptor comprising an amino acid sequence comprising SEQ ID NOS: 16-28 and SEQ ID NOS: 82-113,

wherein the angiogenesis-inhibiting protein has a molecular weight of approximately 18 to approximately 20 kilodaltons as determined by non-reducing and reducing gel electrophoresis, and having an amino acid sequence substantially similar to that of an C-terminal fragment of a collagen molecule.

8. A nucleic acid coding the receptor of Claim 7 having a nucleotide sequence selected from the group consisting of SEQ ID NOS: 56-68.

9. The angiogenesis-inhibiting protein receptor of Claim 7 wherein the receptor comprises laminin protein, tropomyosin, and active fragments and homologs thereof.

10. A method of increasing angiogenesis in an individual, comprising administering to an individual an angiogenesis increasing amount of a receptor of Claim 7.

11. A method of identifying an angiogenesis-inhibiting protein receptor comprising:

(a) obtaining a peptide comprising an amino acid sequence comprising SEQ ID NOS: 16-28 and SEQ ID NOS: 82-113;

(b) testing said peptide for an ability to bind angiogenesis-inhibiting protein,

(c) testing said peptide for an ability to affect angiogenesis-inhibiting protein-mediated angiogenesis, wherein the ability to bind an angiogenesis-inhibiting protein and affect angiogenesis-inhibiting protein-mediated angiogenesis identifies the angiogenesis-inhibiting protein receptor.

(d) wherein the angiogenesis-inhibiting protein has a molecular weight of approximately 18 to approximately 20 kilodaltons as determined by non-reducing and reducing gel electrophoresis, and having an amino acid sequence substantially similar to that of an C-terminal fragment of a collagen molecule.

12. A method of expressing a nucleic acid selected from the group consisting of SEQ ID NOS: 43-80 comprising transfecting the nucleic acid into a vector and host cell system capable of recombinantly producing the protein coded thereby.

13. The method of Claim 11 wherein the angiogenesis-inhibiting protein receptor comprises laminin protein, tropomyosin, and active fragments and homologs thereof.

FIGURE 1

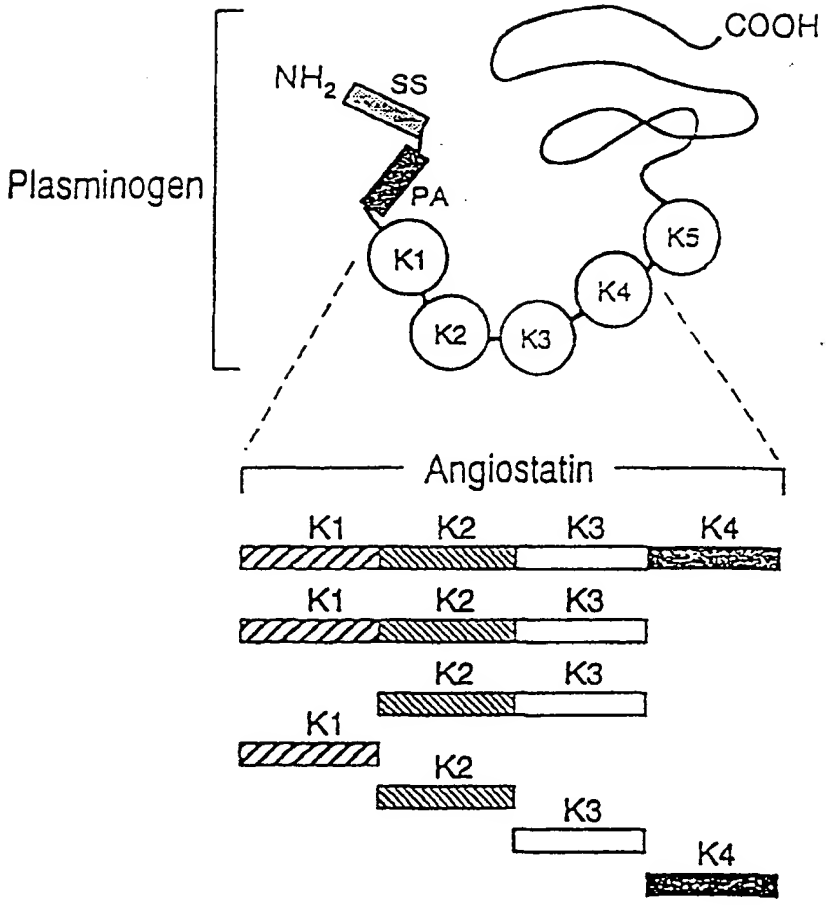
Plasminogen
NH2
SS
PA
COOH
K1
K2
K3
K4
K5
Angiostatin
K1 K2 K3 K4
K1 K2 K3
K2 K3
K1
K2
K3
K4

FIGURE 2

Leu Phe Glu Lys Lys Val Tyr Leu Ser Glu Cys Lys Thr Gly Asn Gly Lys Asn Tyr Arg Gly Thr Met Ser Lys Thr Lys Asn Gly Ile Thr Cys Gln Lys Trp Ser Ser Thr Ser Pro His Arg Pro Arg Phe Ser Pro Ala Thr His Pro Ser Glu Gly Leu Glu Glu Asn Tyr Cys Arg Asn Pro Asp Asn Asp Pro Gln Gly Pro Trp Cys Tyr Thr Thr Asp Pro Glu Lys Arg Tyr Asp Tyr Cys Asp Ile Leu Glu Cys Glu Glu Glu Cys Met His Cys Ser Gly Glu Asn Tyr Asp Gly Lys Ile Ser Lys Thr Met Ser Gly Leu Glu Cys Gln Ala Trp Asp Ser Gln Ser Pro His Ala His Gly Tyr Ile Pro Ser Lys Phe Pro Asn Lys Asn Leu Lys Lys Asn Tyr Cys Arg Asn Pro Asp Arg Glu Leu Arg Pro Trp Cys Phe Thr Thr Asp Pro Asn Lys Arg Trp Glu Leu Cys Asp Ile Pro Arg Cys Thr Thr Pro Pro Pro Ser Ser Gly Pro Thr Tyr Gln Cys Leu Lys Gly Thr Gly Glu Asn Tyr Arg Gly Asn Val Ala Val Thr Val Ser Gly His Thr Cys Gln His Trp Ser Ala Gln Thr Pro His Thr His Asn Arg Thr Pro Glu Asn Phe Pro Cys Lys Asn Leu Asp Glu Asn Tyr Cys Arg Asn Pro Asp Gly Lys Arg Ala Pro Trp Cys His Thr Thr Asn Ser Gln Val Arg Trp Glu Tyr Cys Lys Ile Pro Ser Cys Asp Ser Ser Pro Val Ser Thr Glu Gln Leu Ala Pro Thr Ala Pro Pro Glu Leu Thr Pro Val Val Gln Asp Cys Tyr His Gly Asp Gly Gln Ser Tyr Arg Gly Thr Ser Ser Thr Thr Thr Thr Gly Lys Lys Cys Gln Ser Trp Ser Ser Met Thr Pro His Arg His Gln Lys Thr Pro Glu Asn Tyr Pro Asn Ala Gly Leu Thr Met Asn Tyr Cys Arg Asn Pro Asp Ala Asp Lys Gly Pro Trp Cys Phe Thr Thr Asp Pro Ser Val Arg Trp Glu Tyr Cys Asn Leu Lys Lys Cys Ser Gly Thr Glu Ala Ser Val Val Ala Pro Pro Pro Val Val Leu Leu

FIGURE 3

His Ser His Arg Asp Phe Gln Pro Val Leu His Leu Val Ala Leu Asn Ser Pro Leu Ser Gly Gly Met Arg Gly Ile Arg Gly Ala Asp Phe Gln Cys Phe Gln Gln Ala Arg Ala Val Gly Leu Ala Gly Thr Phe Arg Ala Phe Leu Ser Ser Arg Leu Gln Asp Leu Tyr Ser Ile Val Arg Arg Ala Asp Arg Ala Ala Val Pro Ile Val Asn Leu Lys Asp Glu Leu Leu Phe Pro Ser Trp Glu Ala Leu Phe Ser Gly Ser Glu Gly Pro Leu Lys Pro Gly Ala Arg Ile Phe Ser Phe Asp Gly Lys Asp Val Leu Arg His Pro Thr Trp Pro Gln Lys Ser Val Trp His Gly Ser Asp Pro Asn Gly Arg Arg Leu Thr Glu Ser Tyr Cys Glu Thr Trp Arg Thr Glu Ala Pro Ser Ala Thr Gly Gln Ala Ser Ser Leu Leu Gly Gly Arg Leu Leu Gly Gln Ser Ala Ala Ser Cys His His Ala Tyr Ile Val Leu Cys Ile Glu Asn Ser Phe Met Thr Ala Ser Lys

FIGURE 4

NM226 PAL phage Angiostatin and Plasminogen binding by ELISA

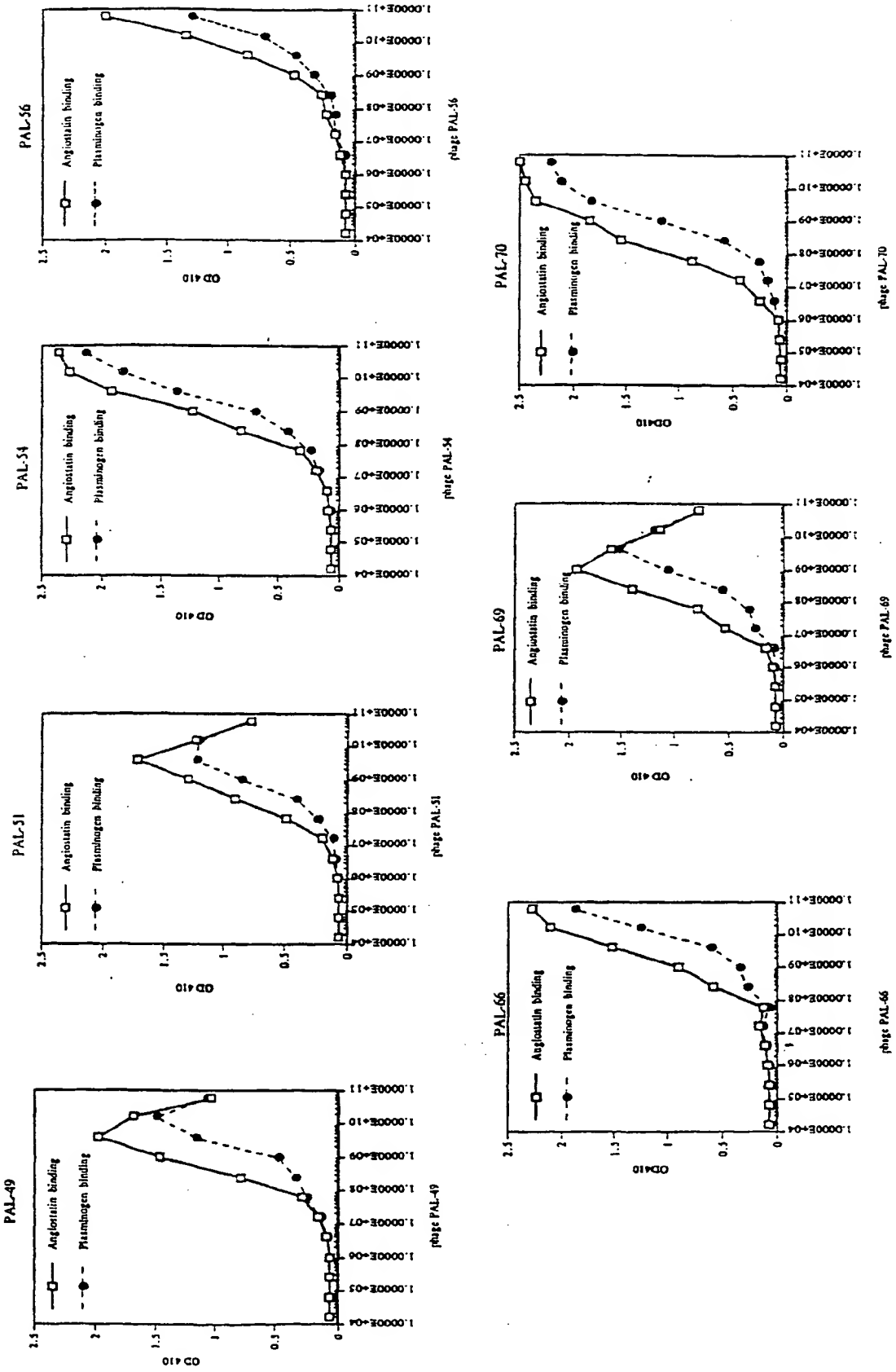

FIGURE 5

NM226 PAC phage Angiostatin and Plasminogen binding by ELISA

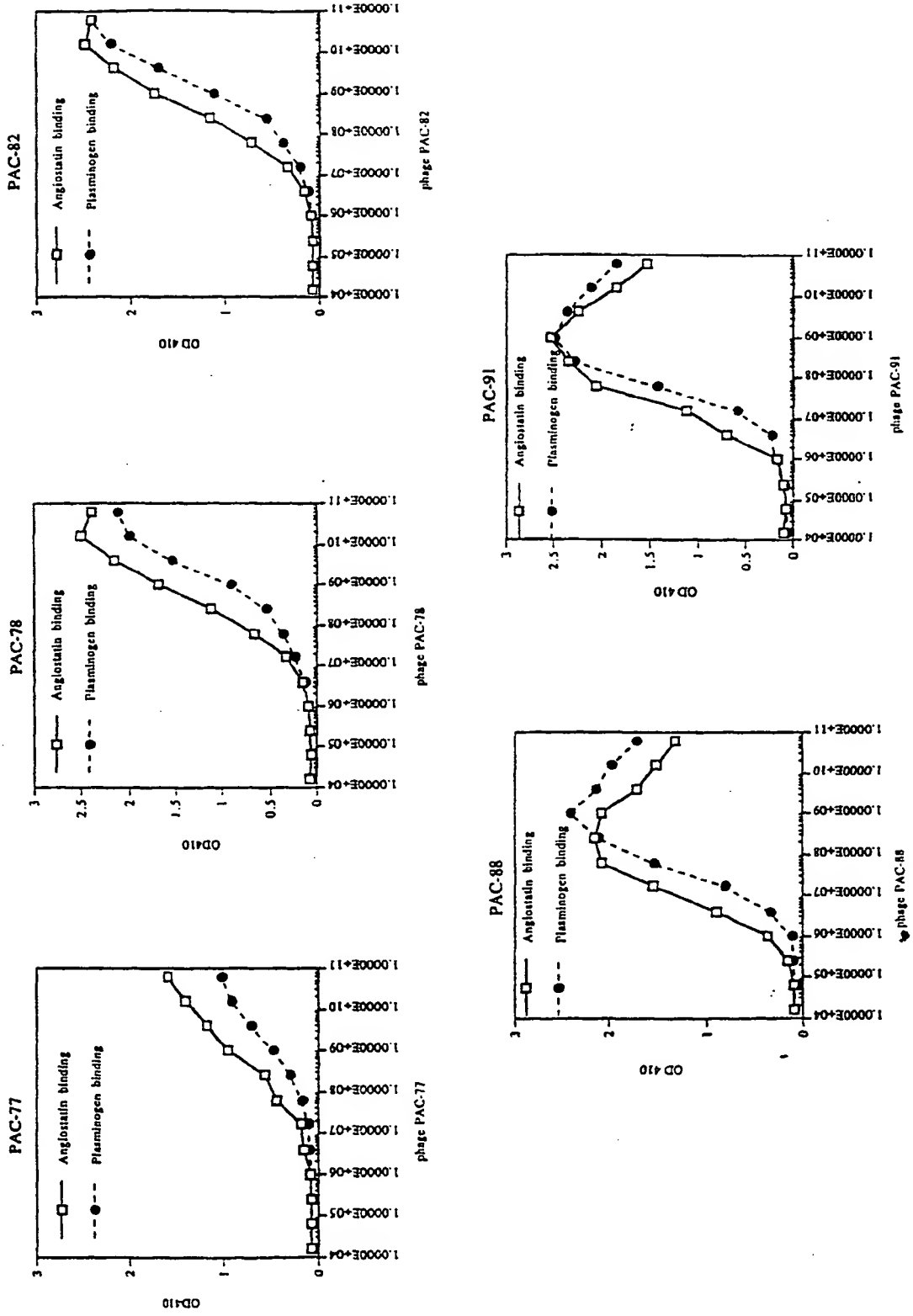

FIGURE 6 A

MGLLQLLAFS FLALCRARVR AQEPEFSYGC AEGSCYPATG DLLIGRAQKL SVTSTCGLHK
PEPYCIVSHL QEDKKCFICN SQDPYHETLN PDSHLIENVV TTFAPNRLKI WWQSENGVEN
VTIQLDLEAE FHFTHLIMTF KTFRPAAMLI ERSSDFGKTW GVYRYFAYDC EASFPGISTG
PMKKVDDIIC DSRYSDIEPS TEGEVIFRAL DPAFKIEDPY SPRIQNLLKI TNLRIKFVKL
HTLGDNLLDS RMEIREKYYY AVYDMVVRGN CFCYGHASEC APVDGFNEEV EGMVHGHCMC
RHNTKGLNCE LCMDFYHDLP WRPAEGRNSN ACKKCNCNEH SISCHFDMAV YLATGNVSGG
VCDDCQHNTM GRNCEQCKPF YYQHPERDIR DPNFCERCTC DPAGSQNEGI CDSYTDFSTG
LIAGQCRCKL NVEGEHCDVC KEGFYDLSSE DPFGCKSCAC NPLGTIPGGN PCDSETGHCY
CKRLVTGQHC DQCLPEHWGL SNDLDGCRPC DCDLGGALNN SCFAESGQCS CRPHMIGRQC
NEVEPGYYFA TLDHYLYEAE EANLGPGVSI VERQYIQDRI PSWTGAGFVR VPEGAYLEFF
IDNIPYSMEY DILIRYEPQL PDHWEKAVIT VQRPGRIPTS SRCGNTIPDD DNQVVSLSPG
SRYVVLPRPV CFEKGTNYTV RLELPQYTSS DSDVESPYTL IDSLVLMPYC KSLDIFTVGG
SGDGVVTNSA WETFQRYRCL ENSRSVVKTP MTDVCRNIIF SISALLHQTG LACECDPQGS
LSSVCDPNGG QCQCRPNVVG RTCNRCAPGT FGFGPSGCKP CECHLQGSVN AFCNPVTGQC
HCFQGVYARQ CDRCLPGHWG FPSCQPCQCN GHADDCDPVT GECLNCQDYT MGHNCERCLA
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GYFGNPSEVG GSCQPCQCHN NIDTTDPEAC DKETGRCLKC LYHTEGEHCQ FCRFGYYGDA
LRQDCRKCVC NYLGTVQEHC NGSDCQCDKA TGQCLCLPNV IGQNCDRCAP NTWQLASGTG
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ITKYFQMSLE AEERVNASTT EPNSTVEQSA LMRDRVEDVM MERESQFKEK QEEQARLLDE
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LQHSAADIAR AEMLLEEAKR ASKSATDVKV TADMVKEALE EAEKAQVAAE KAIKQADEDI
QGTQNLLTSI ESETAASEET LFNASQRISE LERNVEELKR KAAQNSGEAE YIEKVVYTVK
QSAEDVKKTL DGELDEKYKK VENLIAKKTE ESADARRKAE MLQNEAKTLL AQANSKLQLL
KDLERKYEDN QRYLEDKAQE LARLEGEVRS LLKDISQKVA VYSTCL

FIGURE 6B

```
   1 cccggagcag ggcgagagct cgcgtcgccg gaaaggaaga cgggaagaaa gggcaggcgg
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2401 atttctgccc tgttacacca gacaggcctg gcttgtgaat gcgacctca gggttcgtta
2461 agttccgtgt gtgatcccaa cggaggccag tgccagtgcc ggcccaacgt ggttggaaga
2521 acctgcaaca gatgtgcacc tggaactttt ggctttggcc ccagtggatg caaaccttgt
2581 gagtgccatc tgcaaggatc tgtcaatgcc ttctgcaatc ccgtcactgg ccagtgccac
2641 tgtttccagg gagtgtatgc tcggcagtgt gatcggtgct tacctgggca ctggggcttt
2701 ccaagttgcc agccctgcca gtgcaatggc cacgccgatg actgcgaccc agtgactggg
2761 gagtgcttga actgccagga ctacaccatg ggtcataact gtgaaaggtg cttggctggt
2821 tactatggcg accccatcat tgggtcaggt gatcactgcc gcccttgccc ttgcccagat
2881 ggtcccgaca gtggacgcca gtttgccagg agctgctacc aagatcctgt tactttacag
2941 cttgcctgtg tttgtgatcc tggatacatt ggttccagat gtgacgactg tgcctcagga
3001 tactttggca atccatcaga agttgggggg tcgtgtcagc cttgccagtg tcacaacaac
3061 attgacacga cagacccaga agcctgtgac aaggagactg ggaggtgtct caagtgcctg
3121 taccacacgg aaggggaaca ctgtcagttc tgccggtttg gatactatgg tgatgccctc
3181 cggcaggact gtcgaaagtg tgtctgtaat tacctgggca ccgtgcaaga gcactgtaac
3241 ggctctgact gccagtgcga caaagccact ggtcagtgct tgtgtcttcc taatgtgatc
3301 gggcagaact gtgaccgctg tgcgcccaat acctggcagc tggccagtgg cactggctgt
```

FIGURE 6B

```
3361 gacccatgca actgcaatgc tgctcattcc ttcgggccat cttgcaatga gttcacgggg
3421 cagtgccagt gcatgcctgg gtttggaggc cgcacctgca gcgagtgcca ggaactcttc
3481 tggggagacc ccgacgtgga gtgccgagcc tgtgactgtg accccagggg cattgagacg
3541 ccacagtgtg accagtccac gggccagtgt gtctgcgttg agggtgttga gggtccacgc
3601 tgtgacaagt gcacgcgagg gtactcgggg gtcttccctg actgcacacc ctgccaccag
3661 tgctttgctc tctgggatgt gatcattgcc gagctgacca acaggacaca cagattcctg
3721 gagaaagcca aggccttgaa gatcagtggt gtgatcgggc cttaccgtga gactgtggac
3781 tcggtggaga ggaaagtcag cgagataaaa gacatcctgg cgcagagccc cgcagcagag
3841 ccactgaaaa acattgggaa tctctttgag gaagcagaga aactgattaa agatgttaca
3901 gaaatgatgg ctcaagtaga agtgaaatta tctgacacaa cttcccaaag caacagcaca
3961 gccaaagaac tggattctct acagacagaa gccgaaagcc tagacaacac tgtgaaagaa
4021 cttgctgaac aactggaatt tatcaaaaac tcagatattc ggggtgcctt ggatagcatt
4081 accaagtatt tccagatgtc tcttgaggca gaggagaggg tgaatgcctc caccacagaa
4141 cccaacagca ctgtggagca gtcagccccc atgagagaca gagtagaaga cgtgatgatg
4201 gagcgagaat cccagttcaa ggaaaaacaa gaggagcagg ctcgcctcct tgatgaactg
4261 gcaggcaagc tacaaagcct agacctttca gccgctgccg aaatgacctg tggaacaccc
4321 ccaggggcct cctgttccga gactgaatgt ggcgggccaa actgcagaac tgacgaagga
4381 gagaggaagt gtgggggccc tggctgtggt ggtctggtta ctgttgcaca caacgcctgg
4441 cagaaagcca tggacttgga ccaagatgtc ctgagtgccc tggctgaagt ggaacagctc
4501 tccaagatgg tctctgaagc aaaactgagg gcagatgagg caaaacaaag tgctgaagac
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4681 gaagcagttg ctaatgaagt attgaaaatg gagatgccta gcaccccaca gcagttacag
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5281 ctacaaaatg aagcaaaaac tcttttagct caagcaaata gcaagctgca actgctcaaa
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5401 gcaagactgg aaggagaagt ccgttcactc ctaaaggata taagccagaa agttgctgtg
5461 tatagcacat gcttgtaaca gaggagaata aaaaatggct gaggtgaaca aggtaaaaca
5521 actacatttt aaaaactgac ttaatgctct tcaaaataaa acatcaccta tttaatgttt
5581 ttaatcacat tttgtatgag ttaaataaag ccc
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FIGURE 7

```
1021 lrqdcrkcvc nylgtvqehc ngsdcqcdka tgqclclpnv igqncdrcap ntwqlasgtg
1081 cdpcncnaah sfgpscneft gqcqcmpgfg grtcsecqel fwgdpdvecr acdcdprgie
1141 tpqcdqstgq cvcvegvegp rcdkctrgys gvfpdctpch qcfalwdvii aeltnrthrf
1201 lekakalkis gvigpyretv dsverkvsei kdilaqspaa eplknignlf eeaeklikdv
1261 temmaqvevk lsdttsqsns takeldslqt eaesldntvk elaeqlefik nsdirgalds
1321 itkyfqmsle aeervnastt epnstveqsa lmrdrvedvm meresqfkek qeeqarllde
                  ervnddt     wsleqsk
                  drsqaik     wslettk
                 ldranvf      wslehqk
                              wtlestk

1381 lagklqsldl saaaemtcgt ppgascsete cggpncrtde gerkcggpgc qglvtvahna
       gtlqvls

1441 wqkamdldqd vlsalaeveq lskmvseakl radeakqsae dillktnatk ekmdksneel
1501 rnlikqirnf ltqdsadlds ieavanevlk mempstpqql qnltedirer veslsqvevi
1561 lqhsaadiar aemlleeakr asksatdvkv tadmvkeale eaekaqvaae kaikqadedi
1621 qgtqnlltsi esetaaseet lfnasqrise lernveelkr kaaqnsgeae yiekvvytvk
1681 qsaedvkktl dgeldekykk venliakkte esadarrkae mlqneaktll aqansklqll
1741 kdlerkyedn qryledkaqe larlegevrs llkdisqkva vystcl
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FIGURE 8

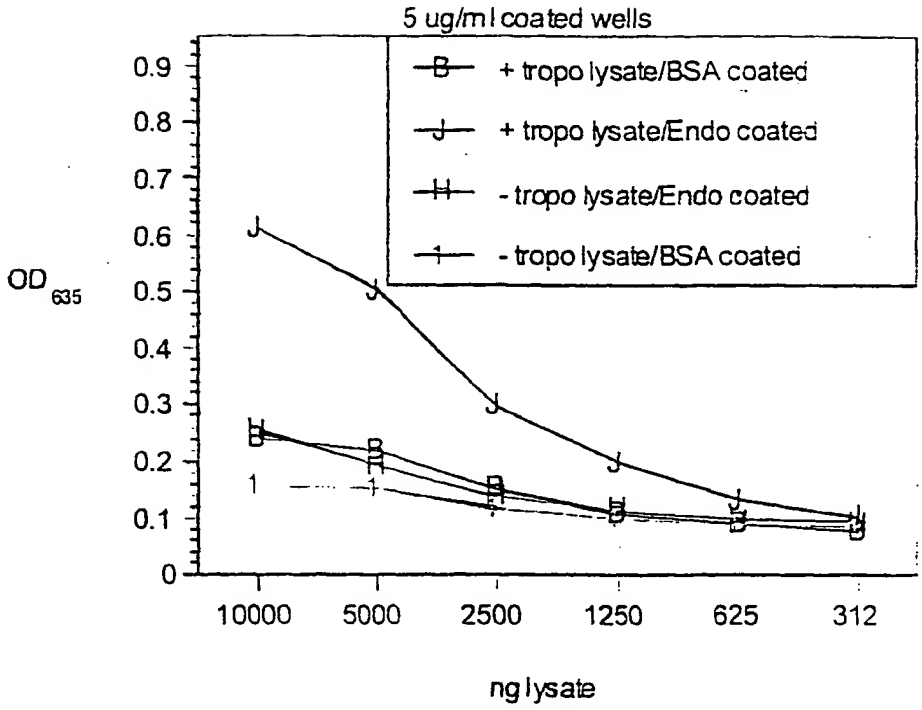
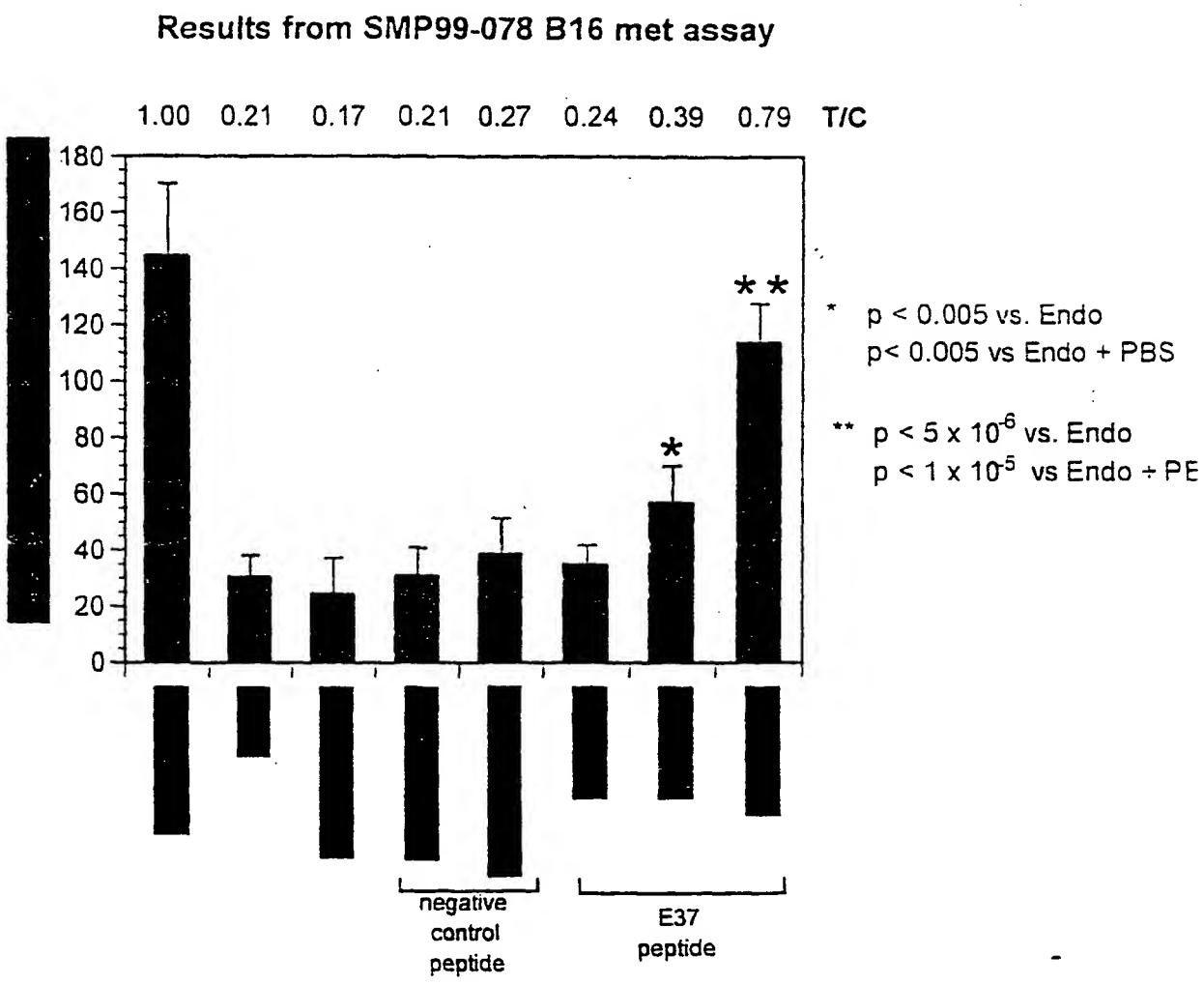

5 ug/ml coated wells
+ tropo lysate/BSA coated
+ tropo lysate/Endo coated
- tropo lysate/Endo coated
- tropo lysate/BSA coated
OD 635
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10000
5000
2500
1250
625
312
ng lysate

FIGURE 9

	CP/PBS buffer	Endo (129)	Endo + PBS	50x control peptide	250x control peptide	10x E37peptide	50x E37 peptide	250x E37 peptide
Lung 1 (# of mets)	126	19	22	27	56	44	74	109
Lung 2 (# of mets)	184	33	20	36	22	37	52	117
Lung 3 (# of mets)	141	27	38	19	31	29	66	98
Lung 4 (# of mets)	117	41	35	26	46	28	51	136
Lung 5 (# of mets)	152	30	5	45	37		41	108
Mean # of mets	144	30	24	30.6	38.4	34.5	56.8	113.6
Stdev	26.10555496	8.06225775	13.2098448	10.06479011	13.16434579	7.505553499	13.10343466	14.22322045
T/C	1	0.20833333	0.16666667	0.2125	0.266666667	0.239583333	0.394444444	0.788888889
T-test, 2 tail								
vs Endo	1.42119E-05	1	0.41122773	0.575498587	0.25837389	0.419869073	**0.004576053**	3.09583E-06
vs CP/PBS	1	1.4212E-05	1.613E-05	5.68215E-05	4.07786E-05	8.92357E-05	0.000156637	0.051541294
vs Endo + PBS	1.61297E-05	0.41122773	1	0.695969254	0.122519263	0.202399998	**0.004285012**	**6.69945E-06**

FIGURE 10


Results from SMP99-078 B16 met assay
1.00 0.21 0.17 0.21 0.27 0.24 0.39 0.79 T/C
180
160
140
120
100
80
60
40
20
0
* *
*
negative control peptide
E37 peptide
* p < 0.005 vs. Endo
p< 0.005 vs Endo + PBS
** p < 5 x 10-6 vs. Endo
p < 1 x 10-5 vs Endo + PE

SEQUENCE LISTING

<110> MacDonald, Nicholas
Sim, Kim Lee

<120> Angiogenesis-Inhibiting Protein Binding Peptides and Proteins and Methods of Use

<130> 05213-0370

<140>
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<210> 1
<211> 378
<212> PRT
<213> Homo sapiens

<400> 1

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Leu Phe Glu Lys Lys Val Tyr Leu Ser Glu Cys Lys Thr Gly Asn Gly
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Lys Asn Tyr Arg Gly Thr Met Ser Lys Thr Lys Asn Gly Ile Thr Cys
            20                  25                  30

Gln Lys Trp Ser Ser Thr Ser Pro His Arg Pro Arg Phe Ser Pro Ala
        35                  40                  45

Thr His Pro Ser Glu Gly Leu Glu Glu Asn Tyr Cys Arg Asn Pro Asp
    50                  55                  60

Asn Asp Pro Gln Gly Pro Trp Cys Tyr Thr Thr Asp Pro Glu Lys Arg
65                  70                  75                  80

Tyr Asp Tyr Cys Asp Ile Leu Glu Cys Glu Glu Glu Cys Met His Cys
                85                  90                  95

Ser Gly Glu Asn Tyr Asp Gly Lys Ile Ser Lys Thr Met Ser Gly Leu
            100                 105                 110

Glu Cys Gln Ala Trp Asp Ser Gln Ser Pro His Ala His Gly Tyr Ile
        115                 120                 125

Pro Ser Lys Phe Pro Asn Lys Asn Leu Lys Lys Asn Tyr Cys Arg Asn
```

```
    130                 135                 140

Pro Asp Arg Glu Leu Arg Pro Trp Cys Phe Thr Thr Asp Pro Asn Lys
145                 150                 155                 160

Arg Trp Glu Leu Cys Asp Ile Pro Arg Cys Thr Thr Pro Pro Pro Ser
                165                 170                 175

Ser Gly Pro Thr Tyr Gln Cys Leu Lys Gly Thr Gly Glu Asn Tyr Arg
            180                 185                 190

Gly Asn Val Ala Val Thr Val Ser Gly His Thr Cys Gln His Trp Ser
        195                 200                 205

Ala Gln Thr Pro His Thr His Asn Arg Thr Pro Glu Asn Phe Pro Cys
    210                 215                 220

Lys Asn Leu Asp Glu Asn Tyr Cys Arg Asn Pro Asp Gly Lys Arg Ala
225                 230                 235                 240

Pro Trp Cys His Thr Thr Asn Ser Gln Val Arg Trp Glu Tyr Cys Lys
                245                 250                 255

Ile Pro Ser Cys Asp Ser Ser Pro Val Ser Thr Glu Gln Leu Ala Pro
            260                 265                 270

Thr Ala Pro Pro Glu Leu Thr Pro Val Val Gln Asp Cys Tyr His Gly
        275                 280                 285

Asp Gly Gln Ser Tyr Arg Gly Thr Ser Ser Thr Thr Thr Thr Gly Lys
    290                 295                 300

Lys Cys Gln Ser Trp Ser Ser Met Thr Pro His Arg His Gln Lys Thr
305                 310                 315                 320

Pro Glu Asn Tyr Pro Asn Ala Gly Leu Thr Met Asn Tyr Cys Arg Asn
                325                 330                 335

Pro Asp Ala Asp Lys Gly Pro Trp Cys Phe Thr Thr Asp Pro Ser Val
            340                 345                 350

Arg Trp Glu Tyr Cys Asn Leu Lys Lys Cys Ser Gly Thr Glu Ala Ser
        355                 360                 365

Val Val Ala Pro Pro Pro Val Val Leu Leu
    370                 375
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<210> 2
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<213> Homo sapiens

<400> 2

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Ser Pro Leu Ser Gly Gly Met Arg Gly Ile Arg Gly Ala Asp Phe Gln
             20                  25                  30

Cys Phe Gln Gln Ala Arg Ala Val Gly Leu Ala Gly Thr Phe Arg Ala
         35                  40                  45

Phe Leu Ser Ser Arg Leu Gln Asp Leu Tyr Ser Ile Val Arg Arg Ala
     50                  55                  60

Asp Arg Ala Ala Val Pro Ile Val Asn Leu Lys Asp Glu Leu Leu Phe
 65                  70                  75                  80

Pro Ser Trp Glu Ala Leu Phe Ser Gly Ser Glu Gly Pro Leu Lys Pro
                 85                  90                  95

Gly Ala Arg Ile Phe Ser Phe Asp Gly Lys Asp Val Leu Arg His Pro
            100                 105                 110

Thr Trp Pro Gln Lys Ser Val Trp His Gly Ser Asp Pro Asn Gly Arg
        115                 120                 125

Arg Leu Thr Glu Ser Tyr Cys Glu Thr Trp Arg Thr Glu Ala Pro Ser
    130                 135                 140

Ala Thr Gly Gln Ala Ser Ser Leu Leu Gly Gly Arg Leu Leu Gly Gln
145                 150                 155                 160

Ser Ala Ala Ser Cys His His Ala Tyr Ile Val Leu Cys Ile Glu Asn
                165                 170                 175

Ser Phe Met Thr Ala Ser Lys
            180
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<400> 3
Glu Arg Val Asn Asp Asp Thr Gly Gly Gly Ser
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<210> 4
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Asp Arg Ser Gly Ala Ile Lys Gly Gly Gly Ser
1 5 10

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<400> 5
Leu Asp Arg Ala Asn Val Phe Gly Gly Gly Ser
1 5 10

<210> 6
<211> 11
<212> PRT
<213> Artificial Sequence

<220>
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<400> 6
Ser Pro Leu Gly Gly Ser Glu Gly Gly Gly Ser
1 5 10

<210> 7

<211> 11
<212> PRT
<213> Artificial Sequence

<220>
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<400> 7
His Ala Ile Tyr Pro Arg His Gly Gly Gly Ser
1 5 10

<210> 8
<211> 13
<212> PRT
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<220>
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Cys Trp Ser Tyr Glu Trp Ser Lys Cys Gly Gly Gly Ser
1 5 10

<210> 9
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<212> PRT
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1 5 10

<210> 10
<211> 13
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binding peptides

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1 5 10

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<400> 11
Cys Trp Ser Leu Glu Thr Thr Lys Cys Gly Gly Gly Ser
1 5 10

<210> 12
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<213> Artificial Sequence

<220>
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<400> 12
Cys Trp Ser Leu Glu His Gln Lys Cys Gly Gly Gly Ser
1 5 10

<210> 13
<211> 13
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic binding peptides

<400> 13
Cys Trp Ser Leu Glu Ile Leu Lys Cys Gly Gly Gly Ser
1 5 10

<210> 14
<211> 13
<212> PRT
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<220>
<223> Description of Artificial Sequence: synthetic binding peptides

<400> 14
Cys Trp Thr Leu Glu Ser Thr Lys Cys Gly Gly Gly Ser
1 5 10

<210> 15
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<212> PRT
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<220>
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<400> 15
Cys Gly Asp Met Ser Asp Arg Pro Cys Gly Gly Gly Ser
1 5 10

<210> 16
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<212> PRT
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<220>
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<400> 16
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1 5 10

<210> 17
<211> 11
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<220>
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<400> 17
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1 5 10

<210> 18
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<400> 18
Trp His Arg Ser Val Trp Lys Gly Gly Gly Ser
1 5 10

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<212> PRT
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1 5 10

<210> 20
<211> 11
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<400> 20
Phe Thr Glu Pro Thr His Lys Gly Gly Gly Ser

1 5 10

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<211> 11
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<400> 21
Lys Asp Tyr Ala Leu Pro Pro Gly Gly Asp Ser
1 5 10

<210> 22
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<220>
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<400> 22
Ser Lys Ile Ala Pro Ile Met Gly Gly Gly Ser
1 5 10

<210> 23
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<220>
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<400> 23
Trp Arg Gln Thr Arg Lys Asp Gly Gly Gly Ser
1 5 10

<210> 24
<211> 11
<212> PRT

<213> Artificial Sequence

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<400> 24
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1 5 10

<210> 25
<211> 13
<212> PRT
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<220>
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<400> 25
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1 5 10

<210> 26
<211> 13
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<400> 26
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1 5 10

<210> 27
<211> 13
<212> PRT
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1 5 10

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cacagaccta gattctcacc tgctacacac ccctcagagg gactggagga gaactactgc 180
aggaatccag acaacgatcc gcaggggccc tggtgctata ctactgatcc agaaaagaga 240
tatgactact gcgacattct tgagtgtgaa gaggaatgta tgcattgcag tggagaaaac 300
tatgacggca aaatttccaa gaccatgtct ggactggaat gccaggcctg ggactctcag 360
agcccacacg ctcatggata cattccttcc aaatttccaa acaagaacct gaagaagaat 420
tactgtcgta accccgatag ggagctgcgg ccttggtgtt tcaccaccga ccccaacaag 480
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taccagtgtc tgaagggaac aggtgaaaac tatcgcggga atgtggctgt taccgtgtcc 600
gggcacacct gtcagcactg gagtgcacag acccctcaca cacataacag gacaccagaa 660
aactttccct gcaaaaattt ggatgaaaac tactgccgca atcctgacgg aaaaagggcc 720
ccatggtgcc atacaaccaa cagccaagtg cggtgggagt actgtaagat accgtcctgt 780
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<210> 30
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<213> Homo sapiens

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gtgcgccgtg ccgaccgcgc agccgtgccc atcgtcaacc tcaaggacga gctgctgttt 240
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ttctcctttg acggcaagga cgtcctgagg caccccacct ggccccagaa gagcgtgtgg 360
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gcctccaagt ag 552

<210> 31
<211> 11
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<220>
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<400> 31
Lys Cys Cys Tyr Tyr Ala Lys Gly Gly Gly Ser
1 5 10

<210> 32
<211> 11
<212> PRT
<213> Artificial Sequence

<220>
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Lys Cys Cys Tyr Pro Ser Ala Gly Gly Gly Ser
1 5 10

<210> 33
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<223> Description of Artificial Sequence: synthetic binding peptides

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Arg Gln Pro Pro His Leu His Gly Arg Gly Ser
1 5 10

<210> 34
<211> 11
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<400> 34
His Lys Tyr Ile Ser Ala Thr Gly Gly Gly Ser
1 5 10

<210> 35
<211> 11
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic binding peptides

<400> 35
Gly Thr Leu Gln Val Leu Ser Gly Gly Gly Ser
1 5 10

<210> 36
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<220>
<223> Description of Artificial Sequence: synthetic binding peptides

<400> 36
Lys Cys Cys Tyr Ser Val Gly Gly Gly Gly Ser
1 5 10

<210> 37
<211> 11
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<220>
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<400> 37
Met Ser Tyr Gln Trp Ser His Gly Gly Gly Ser
1 5 10

<210> 38
<211> 13
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<220>
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<400> 38
Cys Trp Ser Leu Glu His Ser Lys Cys Gly Gly Gly Ser
1 5 10

<210> 39
<211> 13
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<213> Artificial Sequence

<220>
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<400> 39
Cys Val His Ser Ile Glu Arg Glu Cys Gly Gly Gly Ser
1 5 10

<210> 40
<211> 13
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic binding peptides

<400> 40
Cys Tyr Thr Leu Pro Pro Lys Leu Cys Gly Gly Gly Ser
1 5 10

<210> 41
<211> 13
<212> PRT
<213> Artificial Sequence

<220>
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<400> 41
Cys Trp Ser Tyr Glu Trp Ser Lys Cys Gly Gly Gly Ser
1 5 10

<210> 42
<211> 13
<212> PRT
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<220>
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<400> 42
Cys Trp Ser Leu Glu Trp Gln Lys Cys Gly Gly Gly Ser
1 5 10

<210> 43
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<400> 43
gagcgggtta atgatgattg gggtggaggt tcg 33

<210> 44
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<212> DNA
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gatctgtcgg ttgctattaa gggtggaggt tcg 33

<210> 45
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ctggatcggg ctaatgtgtt tggtggaggt tcg 33

<210> 46
<211> 33
<212> DNA
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tcgccgttgg ggggttctga gggtggaggt tcg 33

<210> 47
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<212> DNA
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<220>
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<400> 47
catgctattt atccgcgtca tggtggaggt tcg 33

<210> 48
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<212> DNA
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<220>
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<212> DNA
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tgttggtcgc ttgagcatta gaagtgcggt ggaggt 36

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<400> 53
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<210> 54
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<210> 55
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<220>
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<400> 55
tgtggggata tgtctgatcg tccttgcggt ggaggt 36

<210> 56
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<212> DNA
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<220>
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<400> 56
cataagcgtc ctcgtaataa tggtggaggt tcg 33

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<220>
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<400> 57
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<210> 58
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<400> 58
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<400> 61
aaggattatg cacttccgcc tggtggagat tcg 33

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tcgaagattg cgcctattat gggtggaggt tcg 33

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<210> 64
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<210> 65
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<400> 65
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<210> 66
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<210> 67
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<220>
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<400> 67
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<210> 68
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<212> DNA
<213> Artificial Sequence

<220>
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<400> 68
tgtgcgccgc cgggtctggc gcggtgcggt ggaggt 36

<210> 69
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic binding peptides

<400> 69
aagtgttgtt attatgctaa gggtggaggt tcg 33

<210> 70
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<210> 71
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<210> 72
<211> 33

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<210> 73
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<210> 74
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<400> 75
atgtcttatc agtggtcgca tggtgga 27

<210> 76
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<212> DNA
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<400> 76
tgttggtctc tggagcattc gaagtgcggt ggaggt 36

<210> 77
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tgttatactt tgcctcctaa gctttgcggt ggaggt 36

<210> 79
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<210> 80
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<212> DNA
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Mouse Plasminogen Sequence: SEQ ID NO: 81

met asp his lys glu val ile leu leu phe leu leu leu leu lys pro gly
gln gly asp ser leu asp gly tyr ile ser thr gln gly ala ser leu phe
ser leu thr lys lys gln leu ala ala gly gly val ser asp cys leu ala
lys cys glu gly glu thr asp phe val cys arg ser phe gln tyr his ser
lys glu gln gln cys val ile met ala glu asn ser lys thr ser ser ile
ile arg met arg asp val ile leu phe glu lys arg val tyr leu ser glu
cys lys thr gly ile gly asn gly tyr arg gly thr met ser arg thr lys
ser gly val ala cys gln lys trp gly ala thr phe pro his val pro asn
tyr ser pro ser thr his pro asn glu gly leu glu glu asn tyr cys arg
asn pro asp asn asp glu gln gly pro trp cys tyr thr thr asp pro asp
lys arg tyr asp tyr cys asn ile pro glu cys glu glu glu cys met tyr
cys ser gly glu lys tyr glu gly lys ile ser lys thr met ser gly leu
asp cys gln ala trp asp ser gln ser pro his ala his gly tyr ile pro
ala lys phe pro ser lys asn leu lys met asn tyr cys his asn pro asp
gly glu pro arg pro trp cys phe thr thr asp pro thr lys arg trp glu
tyr cys asp ile pro arg cys thr thr pro pro pro pro pro ser pro thr
tyr gln cys leu lys gly arg gly glu asn tyr arg gly thr val ser val
thr val ser gly lys thr cys gln arg trp ser glu gln thr pro his arg
his asn arg thr pro glu asn phe pro cys lys asn leu glu glu asn tyr
cys arg asn pro asp gly glu thr ala pro trp cys tyr thr thr asp ser
gln leu arg trp glu tyr cys glu ile pro ser cys glu ser ser ala ser
pro asp gln ser asp ser ser val pro pro glu glu gln thr pro val val
gln glu cys tyr gln ser asp gly gln ser tyr arg gly thr ser ser thr
thr ile thr gly lys lys cys gln ser trp ala ala met phe pro his arg
his ser lys thr pro glu asn phe pro asp ala gly leu glu met asn tyr
cys arg asn pro asp gly asp lys gly pro trp cys tyr thr thr asp pro
ser val arg trp glu tyr cys asn leu lys arg cys ser glu thr gly gly
ser val val glu leu pro thr val ser gln glu pro ser gly pro ser asp
ser glu thr asp cys met tyr gly asn gly lys asp tyr arg gly lys thr
ala val thr ala ala gly thr pro cys gln gly trp ala ala gln glu pro
his arg his ser ile phe thr pro gln thr asn pro arg ala asp leu glu
lys asn tyr cys arg asn pro asp gly asp val asn gly pro trp cys tyr
thr thr asn pro arg lys leu tyr asp tyr cys asp ile pro leu cys ala
ser ala ser ser phe glu cys gly lys pro gln val glu pro lys lys cys
pro gly arg val val gly gly cys val ala asn pro his ser trp pro trp
gln ile ser leu arg thr arg phe thr gly gln his phe cys gly gly thr
leu ile ala pro glu trp val leu thr ala ala his cys leu glu lys ser
ser arg pro glu phe tyr lys val ile leu gly ala his glu glu tyr ile
arg gly leu asp val gln glu ile ser val ala lys leu ile leu glu pro
asn asn arg asp ile ala leu leu lys leu ser arg pro ala thr ile thr
asp lys val ile pro ala cys leu pro ser pro asn tyr met val ala asp
arg thr ile cys tyr ile thr gly trp gly glu thr gln gly thr phe gly
ala gly arg leu lys glu ala gln leu pro val ile glu asn lys val cys
asn arg val glu tyr leu asn asn arg val lys ser thr glu leu cys ala
gly gln leu ala gly gly val asp ser cys gln gly asp ser gly gly pro
leu val cys phe glu lys asp lys tyr ile leu gln gly val thr ser trp
gly leu gly cys ala arg pro asn lys pro gly val tyr val arg val ser
arg phe val asp trp ile glu arg glu met arg asn asn